Surgery and radioimmunotherapy in peritoneal carcinomatosis of colorectal origin

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Surgery and radioimmunotherapy in peritoneal carcinomatosis of colorectal origin

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op het gebied van de Medische Wetenschappen

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door

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Outline of this thesis

Peritoneal carcinomatosis, also known as peritoneal carcinosis or carcinosis peritonei, can be defined as the presence of multiple metastatic implants of carcinoma on the peritoneum. The description 'peritoneal carcinomatosis' was first used by Sampson in 1931, who described peritoneal carcinomatosis in relation to ovarian cancer, probably the malignancy with the highest propensity to disseminate intraperitoneally. In the last 25 years, the peritoneal cavity has been recognized as one of the frequent sites of recurrence in colorectal cancer as well. Until recently, patients with peritoneal carcinomatosis of colorectal origin were considered incurable and treated with palliative chemotherapy and surgery when needed. In the nineteeneighties Paul Sugarbaker, an American surgeon, pioneered a different approach, based on the resection of as much macroscopic disease as possible (cytoreductive surgery) followed by intraperitoneal chemotherapy, frequently under hyperthermic conditions. This aggressive approach was primarily aimed at improving locoregional control of disease. However, to date it has become clear that some patients may be even cured with this approach. The Sugarbaker approach has contributed to a better understanding of peritoneal surface malignancy and probably represents an important step forward in the treatment of peritoneal carcinomatosis of colorectal origin. Still, the majority of patients undergoing cytoreductive surgery and adjuvant intraperitoneal chemotherapy eventually present with recurrent disease, even after complete removal of all macroscopic disease. Other adjuvant treatment modalities are therefore necessary in order to improve the outcome of this particular patient category.

The availability of monoclonal antibodies directed against tumor-associated antigens has offered the possibility to guide cytotoxic agents selectively to tumor cells, while normal tissue are relatively spared. After more than 50 years of research, radioimmunotherapy, using radiolabeled monoclonal antibodies, has been accepted as one of the standard treatments for patients with non-Hodgkin lymphoma. Clinical results of radioimmunotherapy in patients with solid cancers, however, have been disappointing. Still, small volume or minimal residual disease has been recognized as a possibly suitable target for radiolabeled antibodies.

This thesis is based upon the the hypothesis that radioimmunotherapy might be an effective treatment modality for peritoneal carcinomatosis of colorectal origin. Since ideally radioimmunotherapy could be applied in combination with cytoreductive surgery, it was decided to use mouse and rat models of small volume peritoneal carcinomatosis, i.e. resectable intraperitoneal tumor xenografts. To test this hypothesis, the efficacy of radioimmunotherapy was first investigated and optimized in a nude mouse model of peritoneal carcinomatosis. Subsequently, in a rat model the efficacy of radioimmunotherapy when applied as adjuvant treatment after cytoreductive surgery is investigated.

1

Antibody-guided radiation therapy of cancer

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The development and evolution of modern chemotherapy during the second half of the twentieth century has improved the clinical outcome of patients with various forms of cancer.[1] Still, in the far majority of malignancies the efficacy of chemotherapy is very limited, with ninety percent of all drug cures occurring in only 10% of cancer types.[2] For these reasons, the feasibility and efficacy of various forms of immunotherapy has been subject of investigation in both preclinical and clinical research. These include active immunotherapy by means of immunization using components extracted from human tumors, and passive immunotherapy using monoclonal antibodies (MAbs), directed against tumor-associated antigens (TAAs), i.e. antigens that are expressed at higher levels in tumors as compared to normal tissues.[3-5] Both passive and active immunotherapy have been reviewed elsewhere and are beyond the scope of this review. Since the initial results of therapy with naked MAbs were disappointing, MAbs were conjugated to drugs, biologic toxins or radionuclides, in order to achieve a preferential delivery of these toxic agents to tumor lesions, while sparing normal tissues. Results of therapy using MAbs conjugated to drugs or toxins have been reviewed elsewhere.[6,7]

Radioimmunotherapy (RIT) using of MAbs labeled with radionuclides has two major advantages over the application of MAbs conjugated with either drugs or toxins. Firstly, tumor cells not expressing the target antigen can still be sterilized by the so-called crossfire phenomenon, i.e., radiation energy emitted by radionuclides bound to antibodies targeting adjacent tumor cells.[8] Secondly, radionuclides are not subject to multidrug resistance. Although promising results have been obtained in RIT for the treatment of non-Hodgkin lymphoma (NHL),[9] clinical results in patients with solid cancers have been modest.[10]

In this article, a brief overview of the history of RIT is given and relevant aspects of the application of radiolabeled MAbs for the treatment of cancers are discussed. Finally, the results of RIT of NHL and several solid cancers (colorectal cancer, ovarian cancer, breast cancer, and renal cell cancer) are reviewed.

Historical overview of RIT

The first theory on the existence of proteins with specific binding capabilities to pathogenic organisms, thus acting as 'magic bullets', was postulated at the end of the 19th century by the German pathologist Paul Ehrlich in his famous side-chain theory ("Seitenkettentheorie").[11] It took almost 50 years before this concept was translated to tumor biology and treatment by Gorer, who showed that an antitumor serum obtained from black mice was able to suppress the growth of leukemia cells in albino mice.[12] However, the first attempts to use unconjugated MAbs for the treatment of cancer proved to be disappointing. After it had been recognized in 1950 that proteins could be labeled with ^{131}I without significantly altering their immunological specificity, [13] Pressman and Korngold tested the tumor-targeting potential of a ^{131}I-labeled rabbit

antiserum in rats bearing osteosarcoma and confirmed preferential antibody uptake in the tumor xenografts.[14] The first clinical trial investigating the therapeutic efficacy of radiolabeled antibodies was performed in the 1950s by Beierwaltes, who treated fourteen patients with metastatic melanoma with ^{131}I-labeled rabbit antibodies and reported a pathologically confirmed complete remission in one patient.[15] In 1965 Gold and Freeman described the discovery of the carcino-embryonic antigen (CEA), that was expressed both in colon adenocarcinoma as well in fetal colon, but that was almost absent in normal healthy colon.[16] In 1974 Mach et al. were the first to report the feasibility of targeting CEA-expressing human colon cancer xenografts in mice using a radiolabeled polyclonal anti-CEA serum.[17] In the late 1970s Goldenberg et al. successfully targeted colon cancer in patients using a polyclonal goat anti-CEA antiserum.[18] Nowadays, CEA has not only become one of the most extensively used tumor markers in clinical oncology, but also, due to its pronounced expression in various carcinomas, one of the most targeted TAAs in RIT. Interest in the clinical use of antibodies boomed after the development of the hybridoma technique by Kőhler and Milstein in 1975.[19] This technique relies on the fusion of antibody-producing B-lymphocytes, obtained from mice immunized with cancer cell extracts, with malignant myeloma cells (Figure 1). The resulting immortal hybridoma cell lines allowed the production and isolation of pure MAbs of predefined specificity against a single epitope. Moreover, with this method gram quantities of these antibodies could be produced eliminating the batch-to-batch variations, which hampered the development of polyclonal antibodies for clinical use. For the development of the hybridoma technique Kőhler and Milstein were awarded the Nobel Prize in Medicine in 1984. Furthermore, using the same technique, several other TAAs could be idientified, such as Mucin-1 (MUC-1) expressed on the majority of adenocarcinomas, the tumor-associated glycoproteins (TAG-72) expressed on ovarian, breast and colorectal carcinomas and G250 on renal cell carcinomas. Since then, the efficacy of RIT using radiolabeled MAbs has been subject of investigation in virtually every cancer in animal models and/or in patients. Promising results have been obtained in patients with NHL, due to their high intrinsic radiosensitivity, the good access of the radiolabeled MAbs to the tumor cells and the intrinsic anti-tumor activity of the antibodies themselves via antibody dependent cytotoxicity (ADCC), complement dependent cytotoxicity (CDC) and other mechanisms, as summarized by Postema et al.[9] This has resulted in the first registered radiolabeled MAbs directed at the surface antigen CD-20 for the treatment of B-cell NHL (^{90}Y-labeled anti-CD20 MAb Zevalin® (Idee Pharmaceuticals, San Diego, CA, USA) and ^{131}I-labeled anti-CD20 MAb Bexxar® (Corixa, Seattle, WA, USA)).

Antibodies structure and size

Antibodies or immunoglobulin molecules are produced by B-lymphocytes in response to a pathogenic challenge. Immunoglobulins consist of two Fab domains and a Fc

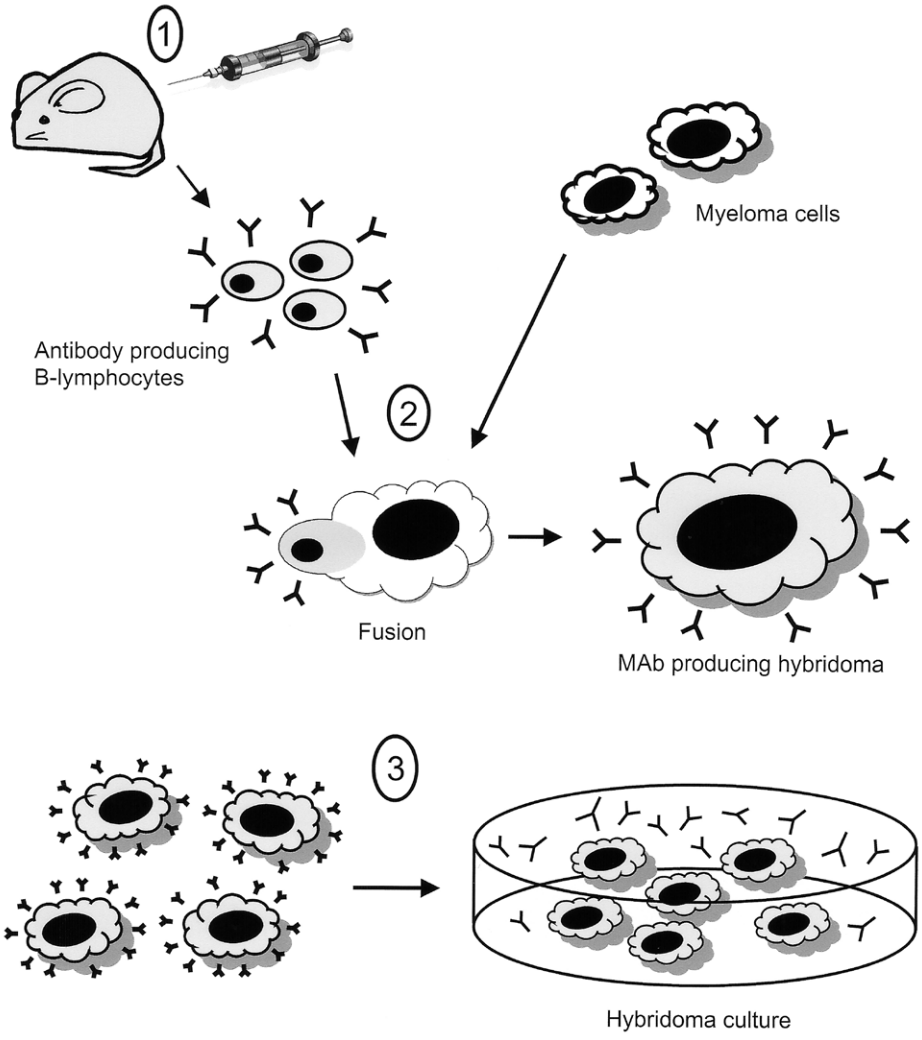

Figure 1

The hybridoma technique by which large quantities of monoclonal antibodies (MAbs) against a particular antigen can be produced.

1. *A mouse is immunized by injection of a particular antigen, in order to stimulate the production of antibodies against this antigen. The antibody forming B-lymphocytes are isolated from the spleen.*
2. *The single antibody-producing B-lymphocytes are fused with malignant myeloma cells, thus creating an immortal cell line. The resulting cell is called a hybridoma.*
3. *Hybridomas producing identical antibodies are allowed to multiply in culture, thus creating an immortal hybridoma cell line that produces MAbs. Adapted from M.J. Koppe et al. Radioimmunotherapy and colorectal cancer. British Journal of Surgery 2005; 93: 264-76. Copyright British Journal of Surgery Society Limited. Reproduced with permission.*

domain, the first containing the variable regions responsible for specific binding to the target antigen of the MAb. The Fc fragment is necessary for activation of the complement cascade or effector cell interaction. There are several subclasses of immunoglobulins, the IgG subclass being the most commonly used in RIT. IgG antibodies are large proteins, with a molecular weight of 150 kDa, which limits the diffusion of the antibodies from the blood into the tumor, resulting in a heterogeneous intratumoral distribution.[20] Furthermore, IgG antibodies are characterized by a long circulatory half-lives in plasma of three to four days. Due to this slow clearance from the blood, tumor-to-background ratios are usually low.[21,22] In order to increase the tumor-to-nontumor ratios of the radiolabel, smaller antibody fragments have been investigated for their tumor-targeting capabilities. These fragments are produced by enzymatic digestion of intact IgG antibodies, and are called F(ab')2 (MW 100 kDa) or F(ab) (MW 40 kDa), as shown in Figure 2. Although the maximum uptake in tumor is reached at earlier time points after intravenous administration and tumor-to-nontumor ratios

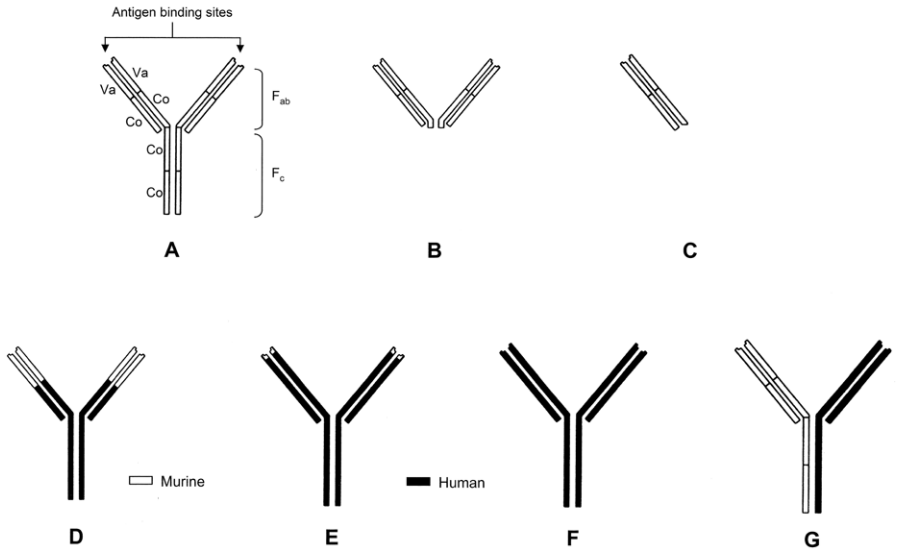

Figure 2

Most important forms of monoclonal antibodies used in clinical radioimmunotherapy.
A *Whole (murine) IgG (MW 150 kDa). Va, variable region; Co, constant region;* **B** *F(ab')2 fragment (MW 100 kDa);* **C** *Fab' fragment (MW 50 kDa);* **D** *Chimeric IgG (67% human). The constant regions of the murine antibody have been replaced by their human analogues;* **E** *Humanized IgG (90-95% human);* **F** *Fully human IgG;* **G** *Bispecific antibody. The antibody, with both arms originating from two separate antibodies, is reactive with two distinct antigens.*
Drawing: M.G. Steffens. Adapted from M.J. Koppe et al. Radioimmunotherapy and colorectal cancer. British Journal of Surgery 2005; 93: 264-76. Copyright British Journal of Surgery Society Limited. Reproduced with permission.

of radiolabeled MAb fragments are, indeed, significantly higher than those obtained with intact IgG MAbs, the absolute uptake in tumor of radiolabeled MAb fragments is usually lower and the tumor retention times are shorter than those of intact MAbs.[23] Furthermore, an important feature of MAb fragments as opposed to intact MAbs is their route of excretion. While intact MAbs are primarily catabolized by the liver and spleen, MAb fragments are mainly excreted via the kidneys, thereby increasing uptake in the kidneys and consequently the kidney absorbed radiation dose.[24]

Immunogenicity

A complicating factor in the application of antibodies is the immune response of the patient against the radiolabeled MAbs. The first MAbs being investigated for radioimmunoscintigraphy and RIT were murine antibodies, which can provoke an immune response in humans. As a result, human-anti-mouse-antibodies (HAMAs) are almost invariably formed, which, upon repeated administration of MAbs to patients, complex the circulating MAbs thereby leading to an accelerated clearance from the blood. As a consequence, the formation of HAMAs generally prevents effective targeting of the tumor after a second administration of the radiolabeled MAb. In order to reduce the immunogenicity of the antibodies, chimeric antibodies were designed combining constant domains of human antibodies with murine variable regions of murine antibodies. Although chimeric antibodies proved to be less immunogenic as compared to murine antibodies, human-anti-chimeric antibodies (HACAs) still develop frequently. In the 1990s humanized antibodies were introduced. Humanized antibodies are almost completely of human origin, with only the complementarity determining regions (CDRs) being murine. With the introduction of phage display libraries or by use of transgenic mice, fully human antibodies can be produced.[25] However, the affinity of antibodies produced by phage display for its antigen is generally lower.

Radionuclides

The most commonly used radionuclides in RIT are beta-emitters, although Auger-electron emitting radionuclides and alpha-emitters can also be used. Beta-particles are electrons that are emitted from the nucleus of an unstable atom. Iodine-131 (^{131}I) and Yttrium (^{90}Y) are the most commonly used beta-emitters in RIT. Rhenium-186 (^{186}Re), Rhenium-188 (^{188}Re), Copper-67 (^{67}Cu) and Lutetium-177 (^{177}Lu) are beta-emitters that have been considered for RIT more recently. These radionuclides differ with respect to physical half-life, the presence or absence of gamma-rays, the energy of the beta-particles and consequently the range of the beta-particles in tissue, as summarized in Table 1. These factors are important with respect to the eventual radiation energy dose that is delivered to the tumor, which can be estimated by dosimetric

analysis.[26] For example, minimal disease consisting of tumor nodules with a diameter of only a few millimeters, theoretically, are not suitable for targeting with ^{90}Y-labeled MAbs, since the energy of the beta-particles is so high that 70% of the radiation energy is deposited outside these small tumors. Indeed, several preclinical studies have shown that medium-energy beta-emitters, such as ^{131}I and ^{177}Lu are more effective for the treatment of small tumor nodules,[27-29] whereas, conversely, high-energy beta-emitters such as ^{90}Y are more suitable for RIT of larger tumors.[29,30]
Another significant factor influencing the tumor-absorbed radiation dose is the fate of the radiolabel after internalization of the radiolabeled MAb into the tumor cell. Internalization of the antibody depends on various factors, including the antibody, the targeted antigen and the tumor cell. However, most antibodies, including those that target antigens located on the surface of the tumor cell, such as anti-CEA antibodies, are eventually catabolized.[31] After internalization of the radiolabeled MAb, the antibody is degraded in the lysosomes. After intralysosomal metabolization of radioiodinated MAbs that are radioiodinated by conventional methods, the radioiodinated tyrosine residues are excreted, thereby reducing the residence time of the radioiodine in the tumor.[32] Radiolabeling of antibodies with ^{90}Y or ^{177}Lu is performed by linking these radionuclides to chelators (DTPA or DOTA), which are chemical moieties that compex free metal ions. These chelators are conjugated to the antibodies and subsequently radiolabeled. After catabolization of MAbs labeled with ^{90}Y- or ^{177}Lu-DTPA/-DOTA, the catabolic products are the radiolabeled chelators conjugated to, in most cases, lysine (e.g. ^{90}Y- or ^{177}Lu-DOTA-lysine). Whereas radioiodinated tyrosine is excreted by the cell, the ^{90}Y- or 177Lu-DTPA/DOTA-lysine metabolites are trapped within the lysosomes, thereby increasing the tumor retention time of these radiolabels.[27]

Dosimetry

Radiation dose calculations, dosing schedules and doses delivered to tumors and normal organs are subject to intensive discussion. There are various ways to determine the radioactivity dose to be administered:

- fixed doses,
- body weight based doses,
- body surface area based doses,
- doses based on the estimated whole body dose, or
- doses based on the estimated red marrow dose.

For almost all radiolabeled MAbs, phase I studies have been conducted to determine the maximum-tolerated dose (MTD). Without exception dose-limiting toxicity consists of leuco- and thrombocytopenia. In general, dosimetric calculations cannot forecast the outcome of therapy, nor can it accurately predict the grade of toxicity following RIT. The need for patient-specific dosimetry is a matter of debate. Some research groups advocate the role of individual dosing based on a diagnostic study for

Table 1. Frequently used radionuclides used in radioimmunotherapy

Radio-nuclide	Half-life	Auger-electron (keV)	ß average (keV)	γ (keV)	Maximum range ß-particles in tissue (mm)	Advantage	Disadvantages
^{131}I	8.0 days	none	192	362	3.0	Easy labeling Inexpensive	High radiation burden to medical personnel/relatives Hospital admittance required
^{90}Y	64 hr	none	935	none	12	High-energy beta-emission Prolonged tumor retention Out-patient treatment possible	Bone-seeking radionuclide No imaging possible
^{186}Re	90.7 hr	none	362	137	5.1	Out-patient treatment possible Ideal gamma for imaging	Laborious labeling
^{188}Re	17.0 hr	none	795 (71%) 729 (25%)	155	27	High-energy	Relatively short half-life
^{177}Lu	6.7 days	none	149	208	2.5	Ideal for small volume disease Prolonged tumor retention	Bone-seeking radionuclide
^{67}Cu	61.9 hr	none	141	185	1.8	Out-patient treatment possible Ideal gamma for imaging	Laborious labeling Limited availability of nuclide Low beta energy
^{125}I	60.1 days	3.2	none	27.5	0.05	Easy labeling	Long half-life

dosimetric purposes. Others showed that RIT can safely be based on body weight of the patient only, without the need for upfront dosimetric calculations before treatment, provided that platelet counts are normal and bone marrow involvement is less than 25%.[33]

Dose-limiting toxicity and strategies to enhance the administered activity dose

Radiation toxicity to radiosensitive normal tissues, especially the bone marrow, limits the activity dose that can be administered safely and consequently, forms a major obstacle that limits the therapeutic efficacy of radiolabeled MAbs. Several strategies have been explored to improve the tumor targeting of radiolabeled MAbs and simultaneously prevent or overcome the dose-limiting bone marrow toxicity. The most important include, 1. the use of antibody fragments, 2. pretargeting strategies, 3. bone marrow support or transplantation.

In order to prevent bone marrow toxicity, the use of antibody fragments has been investigated in various animal models[34-36] and clinical studies.[34,37,38] Antibody fragments generally clear faster from the blood and normal tissues than intact IgG antibodies. However, as mentioned above, the accumulation of radiolabeled antibody fragments in tumor is usually lower. Yet, the use of antibody fragments allows the administration of higher activity doses as compared to intact MAbs.[39]

Another interesting method to improve the tumor targeting of radiolabeled antibodies while simultaneously reducing bone marrow toxicity is the so-called pretargeting approach. In "pretargeted" RIT the radionuclide is administered separately from the tumor targeting antibody. In the first step the unlabeled anti-tumor antibody is administered and allowed to accumulate in the tumor. In a later phase, preferably when the antibody has cleared from the circulation, the radionuclide is administered as a rapidly clearing agent with high affinity for the unlabeled molecule that was injected in the first phase.[40] Preclinical as well as clinical studies have indicated that pretargeted RIT can result in higher tumor-absorbed radiation doses as compared to "classical" RIT, using directly radiolabeled MAbs.[41] However, whereas the radiation dose to the bone marrow is significantly reduced, renal excretion of the radiolabeled hapten results in relatively high radiation dose to the kidneys. Preclinical as well as clinical studies have shown that in pretargeted RIT the kidney-absorbed radiation dose can result in severe glomerulosclerosis and renal failure, which typically occurs several months after therapy.[42]

In pretargeted RIT two main approaches can be distinguished, based on the interaction between the first and second injectate: 1. (strept)avidin and biotin interaction or 2. antibody-hapten interaction. The first approach is based on the extremely avid interaction between (strept)avidin and biotin. The affinity constant (10^{15} M^{-1}) of the (strept)avidin-biotin is 1,000,000-fold higher than that of the average antigen-

antibody interaction. In the first pretargeting studies mice and rabbits with tumor xenografts the target was pretargeted with biotinylated antibodies, after which radiolabeled avidin was administered. These studies provided proof of principle that radiolabeled avidin could accumulate in the biotinylated target.[43-45] It was, however, observed that the radiolabeled avidin also bound to the biotinylated antibody in the circulation. To lower the concentration of the biotinylated antibody in the blood, an 'avidin chase' was given, prior to injection of the radiolabeled avidin, which accelerated the tumor uptake as well as the blood clearance of the radiolabeled (strept)avidin.[46,47] Furthermore, investigators realized soon thereafter that the rapid pharmacokinetics of biotin would be fully exploited, if one would pretarget the tumor with avidin and administer radiolabeled biotin in the last step.[48,49]

The second pretargeting approach is based on the use of so-called bispecific antibodies, i.e. antibodies with affinity to both the tumor-associated antigen as well as a hapten, e.g. a radiolabeled peptide. Due to the relatively low affinity of the hapten for the bispecific MAb (10^9 M^{-1}) as compared to the biotin-avidin interaction (10^{15} M^{-1}), the bispecific MAb-hapten complexes formed in the circulation are relatively labile. As a consequence, the administration of a clearing agent to accelerate the blood clearance of the bispecific MAbs prior to the administration of the radiolabeled hapten is not necessary, which is a major advantage of this pretargeting strategy over avidin-biotin pretargeted RIT. In addition, with the development of humanized bispecific antibody constructs, this method makes us of reagents that will not evoke an antibody response in patients.

Finally, because in RIT using directly radiolabeled the red marrow is the dose-limiting organ, autologous marrow or peripheral blood stem cell (PBSC) or even whole blood reinfusion has been investigated as a means to overcome bone marrow toxicity. Press et al. were the first to investigate the feasibility and efficacy of this approach in patients with NHL, a disease frequently located in the bone marrow. It was shown that autologous PBSC reinfusion allowed the administration of myeloablative doses of RIT in patients with NHL.[50-52] Evidence that reinfusion of autologous PBPCs, or bone marrow can ameliorate the bone marrow suppression and, indeed, modify the myelotoxicity in high-dose RIT was furthermore reported in patients with breast cancer, medullary thyroid cancer, and CRC.[53-56] Colnot et al. demonstrated the feasibility of further dose-escalation of ^{186}Re-based RIT in patients with advanced squamous cell carcinoma of the head and neck by stimulating the bone marrow with granulocyte colony-stimulating factor (G-CSF) and reinfusing one litre of unprocessed whole blood three days after administration of the radiolabeled MAbs.[57] The results indicated that the MTD could be doubled from 1 to 2 GBq/m^2. The latter method, however, is only suitable when radionuclides with relatively short half-lives, such as ^{186}Re or ^{90}Y, are used, since whole blood specimens can be stored for maximally three days.

Clinical results of RIT in NHL

The first report on the treatment of NHL with monoclonal antibodies (MAbs) dates from 1980, when the use of idiotypic MAbs against malignant lymphoma cells in a patient was described.[58] In 1997, rituximab – a chimeric anti-CD20 MAb – became commercially available. Nowadays, it is widely used, and indications are still expanding. Radiolabeled MAbs have been applied successfully in lymphoma treatment. In 2002 and 2003, respectively, two radiolabeled murine anti-CD20 MAbs were approved for clinical use by the FDA. Still, RIT of lymphoma patients is mainly given in trials, in order to define the role of RIT in routine NHL management. An overview of clinical studies with various MAbs and various radionuclides is presented in Table 2.[51,59-77] In the following the representative trials will be discussed.

^{90}Y-ibritumomab (Zevalin®)

^{90}Y-ibritumomab was the first radiopharmaceutical to be approved for the treatment of follicular lymphoma patients who relapse or are refractory following rituximab treatment. Ibritumomab and rituximab are very much alike: Ibritumomab is the murine ancestor of the chimeric MAb rituximab. In the phase I study using ^{90}Y-labeled ibritumomab in 14 patients, RIT was preceded by various amounts of unlabeled ibritimomab. Predosing with unlabeled MAbs resulted in improved biodistribution of the radiolabeled MAb and decreased doses to the spleen and spine.[78] In a subsequent multicenter trial, 50 patients were pretreated with unlabeled rituximab, followed by ^{90}Y-labeled ibritumomab.[76] In that trial the currently used dose of rituximab and ^{90}Y-ibritumomab was determined: 14.8 MBq/kg was chosen if platelet counts were above 150 × 109/l, and a dose of 11.1 MBq/kg if platelet counts ranged between 100 and 150 × 109/l. The infusion of ^{90}Y-ibritumomab was preceded by two infusions of 250 mg/m^2 rituximab with a one-week interval. The overall response rate in this study was 68% (13 CRs and 21 PRs).

In a subsequent phase III trial, RIT with ^{90}Y-ibritumomab was compared with rituximab alone in 143 patients with relapsed or refractory low-grade follicular or transformed CD20-positive NHL.[77] Patients received either a single intravenous dose of 14.8 MBq/kg of ^{90}Y-ibritumomab preceded by two infusions of 250 mg/m^2 rituximab (n = 73), or four weekly doses of 375 mg/m^2 of rituximab (n = 70). The overall response rate was 80% for the ^{90}Y-ibritumomab group versus 56% for the rituximab group. CR rates were 30% and 16% in the RIT and rituximab groups, respectively.

Zevalin® has been approved for clinical use in both the USA and the European Union. Registration in the USA includes imaging using ^{111}In-ibritumomab following the first rituximab infusion. Zevalin's product information mentions that ^{90}Y-ibritumomab should not be administered to patients with altered biodistribution as determined by imaging with ^{111}In-ibritumomab. In the European Union, imaging using ^{111}In-ibritumomab is thought to be unnecessary, although opinions differ.[79]

^{131}I-tositumomab (Bexxar®)

In June 2003, the FDA approved Bexxar® for the treatment of patients with CD20-positive follicular NHL, with and without transformation, whose disease is refractory to rituximab and has relapsed following chemotherapy. Registration was preceded by a randomized open-label multicenter study, comparing the efficacy and safety of ^{131}I-labeled tositumomab to unlabeled tositumomab.[62] Seventy-eight patients were enrolled. Patients receiving ^{131}I-tositumomab therapy were dosed according to protocol, i.e. 450 mg of unlabeled tositumomab and a tacer dose of 185 MBq ^{131}I labeled to 35 mg of tositumomab, followed one to two weeks later by the therapeutic dose of ^{131}I-tositumomab, again preceded by 450 mg of unlabeled tositumomab. The dosing scheme is discussed in more detail later. The patients randomized to unlabeled tositumomab treatment received two doses of 485 mg, i.e. the same total antibody dose that is administered to patients treated with ^{131}I-tositumomab. Confirmed responses were documented in 23 of 42 (55%) patients who received tositumomab RIT (33% CR, 21% PR), and 6 of 36 (17%) patients who received tositumomab (8% CR, 8% PR), respectively. The median duration of confirmed responses for Bexxar®-treated patients has not been reached yet, and for unlabeled tositumomab treated patients it was 18 months. This study documents that the radioiodine component of RIT using ^{131}I-tositumomab provides significant therapeutic effect over and above that provided by unlabeled tositumomab with an acceptable toxicity profile.[62]

As mentioned earlier, the dosing scheme of Bexxar® differs from the dosing scheme of Zevalin®. Whereas the latter uses the patient's body weight to dose the radiopharmaceutical, the former depends on pretherapy dosimetry. In a phase I study, nine patients were treated with one or two doses of ^{131}I-tositumomab, preceded by a tracer dose and by variable amounts of unlabeled antibody to determine whether presaturation of non-specific binding sites or reservoirs of non-malignant B cells would allow better access to tumor sites.[80] Optimal tumor targeting was obtained when administering 685 mg unlabeled tositumomab prior to administration of the radioiodinated MAb. Myelotoxicity occurred when whole-body radiation doses exceeded 0.75 Gy, which was thus defined as the MTD.[81] In the following multicenter phase II trial, all patients received two infusions of 450 mg unlabeled antibody, one preceding the tracer dose, one preceding the therapeutic dose of ^{131}I-tositumomab.[74] Aim of the dosimetric study is to determine the therapeutic amount of activity to be infused, leading to an estimated whole-body dose of 0.75 Gy, if platelet counts are above 150 × 10^9/l. If platelet counts were between 100 and 150 × 10^9/l, the patient should receive less activity, leading to a whole-body dose of 0.65 Gy.

^{90}Y-epratuzumab (LymphoCide Y-90)

Epratuzumab is the only humanized MAb used for RIT of NHL patients. This humanized MAb was produced in order to reduce the immunogenicity of the murine

Table 2. Representative radioimmunotherapy trials in patients with non-Hodgkin lymphoma

Reference	Radiopharmaceutical	Target antigen	MAb type	No. of patients	Myeloablative RIT	Dosing schedule	Responses
DeNardo et al.[63]	^{131}I-Lym-1	HLA-DR10	Murine IgG_{2a}	18	-	multiple	2 CR, 8 PR, 3 SD, 1 PD, 4 AE
Goldenberg et al.[65]	^{131}I-LL2	CD22	Murine IgG_{2a}	7	-	double	2 PR, 3 no R, 2 AE
Czuczman et al.[61]	^{131}I-OKB7	CD21	Murine IgG_{2b}	18	-	quadruple	1 PR, 12 SD
Press et al.[51]	^{131}I-MB-1	CD37	Murine IgG_{1}	6	3/6	RID/RIT	6 CR
	^{131}I-1F5	CD20	Murine IgG_{2a}	1	1/1	RID/RIT	1 PR
	^{131}I-anti-B1	CD20	Murine IgG_{2a}	12	11/12	RID/RIT	10 CR, 1 PD, 1 SD
Juweid et al.[66]	^{131}I-LL2	CD22	Murine IgG_{2a}	7	-	multiple	1 CR, 1 PR, 5 unk
	^{131}I-LL2 F(ab)2	CD22	Murine $F(ab)_{2}$	13	-	multiple	1 CR, 1 PR, 11 unk
	^{131}I-cLL2	CD22	Chimeric IgG_{2a}	1	-	multiple	1 unk
	^{131}I-LL2	CD22	Murine IgG_{2a}	3	3/3	multiple	2 PR, 1 NE
White et al.[75]	^{90}Y-anti-Id			9	-	multiple	2 CR, 1 PR, 3 SD, 3 PD
DeNardo et al.[64]	^{131}I-Lym-1	HLA-DR10	Murine IgG_{2a}	21	-	RID/RIT 1 – 4	7 CR, 4 PR, 9 SD, 1 PD
Behr et al.[59]	^{131}I-hLL2	CD22	Humanized IgG_{2a}	5	2/5	single	2 CR, 1 PR, 1 PD, 1 NA
	^{131}I-C2B8	CD20	Chimeric IgG_{1}	5	5/5	RID/RIT	3 CR, 1 PR, 1 NA

Table 2. Continued

Reference	Radiopharmaceutical	Target antigen	MAb type	No. of patients	Myeloablative RIT	Dosing schedule	Responses
Juweid et al.[67]	^{131}I-hLL2	CD22	Humanized IgG_{2a}	13	-	RID/RIT	1 CR, 1 PR, 5 SD, 6 PD
	^{90}Y-hLL2	CD22	Humanized IgG_{2a}	7	-	RID/RIT	2 PR, 5 PD
Lindén et al.[69]	^{131}I-LL2	CD22	Murine IgG_{2a}	8	-	RID/RIT 1 – 3	3 PR, 1 SD, 4 PD
O'Donnell et al.[70]	^{67}Cu-Lym-1	HLA-DR10	Murine IgG_{2a}	12	-	multiple	1 CR, 6 PR
Witzig et al.[76]	^{90}Y-2B8	CD20	Murine IgG_{1}	50	-	rituximab/RIT	13 CR, 21 PR
Vose et al.[74]	^{131}I-anti-B1	CD20	Murine IgG_{2a}	45	-	RID/RIT	15 CR, 12 PR
Davis et al.[62]	^{131}I-anti-B1	CD20	Chimeric IgG_{1}	42	-	RID/RIT	14 CR, 9 PR
Witzig et al.[77]	^{90}Y-2B8	CD20	Murine IgG_{1}	73	-	rituximab/RIT	22 CR, 36 PR
Behr et al.[60]	^{131}I-C2B8	CD20	Chimeric IgG_{1}	7	7/7	RID/RIT	6 CR, 1 PR
Postema et al.[71]	^{186}Re-hLL2	CD22	Humanized IgG_{2a}	15	-	RID/RIT	1 CR, 4 PR, 4 SD, 6 PD
Turner et al.[73]	^{131}I-C2B8	CD22	Chimeric IgG_{1}	35	-	rituximab/RIT	19 CR, 6 PR
Scheidhauer et al.72	^{131}I-C2B8	CD22	Chimeric IgG_{1}	26	-	RID/RIT	9 CR, 5 PR
				25	25/25	RID/RIT	12 CR, 7 PR
Kaminski et al.80	^{131}I-anti-B1	CD20	Chimeric IgG_{2a}	76	-	RID/RIT	57 CR, 14 PR

RID radioimmunodetection, tracer dose; CR complete remission; PR partial remission; SD stable disease; PD progressive disease; NE not evaluable

anti-CD22 MAb LL2. This antibody has been used in clinical trials labeled with ^{131}I, ^{90}Y, and ^{186}Re. A preclinical study suggested a better biodistribution for ^{90}Y-epratuzumab than for ^{131}I- or ^{186}Re-epratuzumab.[82] In a first clinical study, comparing ^{131}I- and ^{90}Y-labeled epratuzumab, treatment with both radiolabels was equally safe, and pharmacokinetics and dosimetry were similar, but the tumor dosimetry of ^{90}Y-epratuzumab appeared to be more favorable than that of ^{131}I-epratuzumab.[67] ^{90}Y was chosen for further trials. A dose-escalation study showed that treatment with ^{90}Y-epratuzumab was tolerated well at a dose of 0.74 GBq/m^2 by patients who had not been treated with high-dose chemotherapy prior to RIT.[83] In a group of lymphoma patients with high-dose chemotherapy prior to RIT, a dose of 0.37 GBq/m^2 did not lead to dose-limiting toxicity.[83]

Protein doses in all infusions are low: only 0.75 mg epratuzumab per kg body weight is given without any preceding unlabeled antibody infusion. Even low doses of activity are associated with responses. A group at the University Hospital in Lund, Sweden, gave patients two or three injections of only 185 MBq ^{90}Y-epratuzumab/m^2. Patients with prior high-dose chemotherapy and stem cell rescue received only 92.5 MBq/m^2. In five of eight patients, partial or complete responses were observed.[84]

At the Radboud University Nijmegen Medical Center, The Netherlands, we have been studying the use of ^{186}Re-labeled epratuzumab in a dose-escalation study.71 Eighteen patients with CD22-positive NHL were included in this trial, 15 of whom were treated with ^{186}Re-epratuzumab. After inclusion, a tracer dose of 750 MBq ^{99m}Tc-epratuzumab was infused. Whole-body gamma-camera images were made on the day of and one day after infusion. In case of unfavorable biodistribution – predominant uptake in bone marrow and spleen – no RIT was given, as was the case in 2 patients. If a normal biodistribution was seen, patients were treated one week later with ^{186}Re-epratuzumab. The maximum-tolerated dose appeared to be 2.0 GBq/m^2. A CR was observed in 1 of 15 patients treated, 4 patients had a PR lasting several months to more than a year. Four patients had stable disease following one single injection with ^{186}Re-epratuzumab.

Myeloablative RIT

At the University of Washington in Seattle, WA, the group of Press et al. used Bexxar® for the treatment of refractory NHL. They re-infused stem cells after RIT to enable administration of high, myeloablative doses of radiolabeled tositumomab. In aiming at myeloablation, toxicity to organs other than the bone marrow will limit the radiation dose that can be tolerated. Therefore a dose escalation study was designed in which the dose to tumor and critical normal organs was calculated using dosimetry and biopsy specimens after a diagnostic procedure with ^{131}I-labeled MAbs. This study showed that doses less than 27.25 Gy to normal organs did not cause serious, irreversible non-hematological toxicity.[51] In subsequent trials, doses of ^{131}I-tositumomab estimated to deliver 27 Gy to the dose-limiting organ, gener-

ally the lungs, were administered.[85] This myeloablative approach led to complete responses in 30 of 36 patients.[86] A long-term follow-up study of the 29 patients treated with myeloablative doses of Bexxar® has documented estimated overall and progression-free survival rates of 68% and 42%, respectively, with a median follow-up time of 42 months.

Another approach is the combination of myeloablative RIT and high-dose chemotherapy, followed by autologous hematopoietic stem cell rescue. The role of RIT as reported here differs from other forms of RIT. In these cases RIT replaces total body irradiation. The duration of response achieved by this highly effective approach should especially be compared with the duration of response after chemotherapy followed by total body irradiation and stem cell support. In a trial using Bexxar® and etoposide and cyclophosphamide, the overall survival and the progression-free survival of the group treated with myeloablative RIT appear retrospectively to be better than in the historic, conventionally treated control group.[87] Nademanee et al. presented data on the use of Zevalin® in combination with the same chemotherapeutics (etoposide and cyclophosphamide), followed by autologous stem cell transplant.[88] They concluded that the addition of RIT to high-dose chemotherapy did not increase transplant-related toxicity, nor did it delay engraftment. They suggested that myeloablative RIT could replace TBI in this regimen, although further studies and longer follow-up are required.

First-line treatment

In most trials using RIT to treat NHL patients, patients with relapsed of refractory lymphoma were recruited, some of them heavily pretreated. Recently, very encouraging results of first-line treatment of lymphoma patients with radiolabeled antibodies were published.[68] In that study, 76 previously untreated patients with stage III or IV follicular lymphoma were enrolled. They were treated with Bexxar® according to the aforementioned dosing scheme. The overall response rate was as high as 95%, 57 of 76 patients achieving a CR (75%) and 15 a PR (20%). The 5-year progression-free survival (PFS) was 59%, with a median PFS of 6.1 years. Toxicity was very mild, especially when compared to normal first-line treatment: only 4 patients experienced grade IV neutropenia. On the other hand, hypothyroidism was induced in 13-19% of patients. The use of a murine MAb in this untreated group of patients is disadvantageous: HAMA were induced in 48 of 76 patients (63%), possibly prohibiting the use of murine MAbs in case of recurrence.

Although the results are excellent, Connors warns for a possible selection bias.[89] The group treated appears to consist of apparently younger patients with less bulky disease. Therefore, more, preferably randomized trials are needed.

Clinical results of RIT in solid cancers

So far the application of radiolabeled antibodies for the treatment of patients with solid cancers has been less successful than in patients with NHL. This can only partially be attributed to the generally lower sensitivity to radiation of solid cancers. In addition, solid cancers are targeted less efficiently with radiolabeled antibodies than hematological malignancies owing to a combination of tumor-related factors. These include a limited vascular supply, heterogeneous uptake of the antibody in the tumor, and elevated interstitial pressure in combination with a relatively long transport distance in the interstitium.[90] As a consequence, the uptake of radiolabeled antibodies in solid tumors is impaired (generally about 0.001 – 0.01% of the injected dose per gram of tumor) and tumor-absorbed radiation doses usually do not exceed 1,500 cGy in most instances. Still, it has been appreciated that, since the uptake in tumor is inversely correlated with tumor size, minimal residual disease may constitute a suitable target for radiolabeled antibodies. Indeed, RIT has shown to be very effective in animal models of small volume carcinoma and may even be more effective than equally toxic chemotherapy in, for example, experimental colon cancer.[91]

The cancer types that have been targeted in clinical RIT were chosen based on their antigen-expression profile and the availability of antibodies directed against these antigens. The malignancies most commonly targeted in clinical RIT are epithelial cancers, e.g. colorectal cancer, ovarian cancer, medullary thyroid cancer, and, to a lesser extent, breast, prostate and renal cell cancer. The most commonly targeted antigens are the carcinoembryonic antigen (CEA, mainly in colorectal, medullary thyroid and breast cancer), tumor-associated antigen-72 (TAG-72, mainly in colorectal, ovarian and breast cancer), Mucin-1 (MUC-1, mainly in ovarian and breast cancer), and G250 (renal cell cancer). Almost all patient series published to date on the application of RIT for the treatment of solid cancers are derived from phase I/II trials, in which the radiolabeled antibodies were administered in dose-escalating steps with the aim to determine the maximal tolerated dose. As a result, in most studies suboptimal doses were administered in most cases. Furthermore, the patients eligible for these phase I/II RIT trials almost invariably had metastatic, mostly bulky disease, and had been heavily pretreated with chemotherapy and/or radiotherapy. Complete responses have rarely been reported, although in many studies a few patients still experienced minor, partial or mixed responses or stabilization of previously progressive disease. In most trials, ^{131}I or ^{90}Y were the radionuclides applied, although in some trials ^{186}Re, ^{188}Re, or ^{177}Lu were used.

In the following the most important and representative results will be reviewed for RIT of colorectal cancer, ovarian cancer, breast cancer and renal cell cancer. Finally, the trials published to date on the therapeutic efficacy of pretargeted RIT will be summarized.

Colorectal cancer

TAAs targeted in clinical RIT of colorectal cancer include CEA, TAG-72, A33, the colon specific antigen p (CSA-p), and the epithelial cellular adhesion molecule (Ep-CAM, also known as 17-1A).[92] The best representative trials have been summarized in Table 3.

CEA

Since 95% of colorectal carcinomas express CEA, this antigen has been the most targeted antigen in patients with colorectal cancer. To date, fourteen trials have been published reporting the results of ^{131}I-, ^{90}Y- or ^{188}Re-based RIT using seven different anti-CEA antibodies in patients with CRC.

Juweid et al. investigated the pharmacokinetics, immunogenicity and therapeutic efficacy of ^{131}I-labeled F(ab')2 fragments of the murine anti-CEA MAb NP-4 (also known as IMMU-4).[39] Thirteen patients with CEA-expressing disseminated cancers, among whom eight with colorectal cancer, were treated with repeated administrations of ^{131}I-labeled NP-4 (up to 9.4 GBq per administration). Although the majority of patients developed HAMAs after their second administration leading to accelerated clearance of the radiolabeled MAbs, seven patients (among whom four patients with metastatic CRC) showed stabilization of previously progressive disease for up to seven months.[39]

Mittal et al.[93] investigated the toxicity and efficacy of the ^{131}I-labeled NP-4 IgG MAb at 1110 MBq or 2220 MBq/m^2 combined with hyperthermia in six patients with advanced CRC. One patient showed a minor response, two reported symptom improvement whereas in five patients there was a drop in serum CEA levels.

The largest series of patients that were treated with ^{131}I-labeled NP-4 was published by Behr et al.,[94] who tested the antitumor activity of escalating activity doses of ^{131}I-labeled NP-4 IgG in 57 patients with CEA-expressing malignancies (among whom 29 patients with CRC). The activity doses administered were varied between 1628 MBq and 9953 MBq, which were based on dosimetric estimates of the radiation dose to the red marrow. Antitumor effects were seen in twelve of 35 assessable patients, including one partial remission, four mixed/minor responses and seven stabilizations of previously progressive disease. It was not specified which patients responded.

Juweid et al. were the first to test the efficacy of ^{188}Re-based RIT using the murine IgG1 MAb MN-14 in eleven patients with disseminated CEA-positive cancers, among which ten patients with colorectal carcinoma.[95] The murine MN-14 MAb is a second-generation anti-CEA antibody with a ten-fold higher affinity than the NP-4 MAb (1 x 10^9 M^{-1}).[96] Patients were treated with repeated administrations of ^{188}Re-labeled MN-14 at escalating dose levels, varying from 740 MBq till 2886 MBq. The MTD of fractioned doses of ^{188}Re-MN-14 was reached at 2220 MBq/m^2, with red marrow suppression being the only dose-limiting toxicity observed. No therapeutic responses were recorded. The authors concluded that, since high doses of ^{188}Re-labeled MAbs can be administered on an outpatient basis, ^{188}Re might be a suitable radionuclide for RIT.

Table 3. Representative phase I/II radioimmunotherapy trials in patients with colorectal cancer

Reference	Radiopharmaceutical	Target antigen	MAb type	No. of patients	Responses	Special features
Mittal et al.[93]	^{131}I-IMMU-4	CEA	Murine IgG_1	9	1 PR; 2 symptom improvement; 5 drop serum CEA	Combined with hyperthermia
Meredith et al.[109]	^{131}I-COL-1 & ^{131}I-CC-49	CEA/TAG-72	Both murine IgG_1	14	4 SD	Dual-antibody RIT combined with IFNa-2b
Ychou et al.[56]	^{131}I-F6	CEA	Murine F(ab')2	10	1 PR; 2 SD	
Behr et al.[97]	^{131}I-MN-14	CEA	Humanized IgG_1	12	2 PR; 4 Mix/MinR/SD; 9 50% reduction of serum CEA	
Behr et al.[103]	^{131}I-F023C5	CEA	Murine IgG_1	10	1 CR; 2 PR; 4 SD	
Behr et al.[98]	^{131}I-MN-14	CEA	Humanized IgG_1	30	3 PR, 8 MR,	Adjuvant RIT in nine patients after metastasectomy
Wong et al.[101]	^{90}Y-T84.66	CEA	Chimeric IgG_1	21	11 SD; 1 MixR	Combined with 5-fluorouracil
Meredith et al.[108]	^{131}I-B72.3	TAG-72	Chimeric IgG_4	12	3 SD; 1 MinR	
Murray et al.[111]	^{131}I-CC-49	TAG-72	Murine IgG_1	15	None	
Divgi et al.[107]	^{131}I-CC-49	TAG-72	Murine IgG_1	24	6 SD	
Welt et al.[114]	^{131}I-A33	A33	Murine IgG_{2a}	23	3 MixR; 2 minor drops serum CEA	
Welt et al.[115]	^{125}I-A33	A33	Murine IgG_{2a}	21	1 MixR; 12 SD	
Meredith et al.[118]	^{125}I-17-1A	Ep-CAM	Chimeric IgG_{2a}	28	10 SD	

SD stable disease; MR minor response; MixR mixed response; PR partial response; CR complete response

An important disadvantage of ^{188}Re, however, is its short half-life of only 17 hours, which is inefficient when used in combination with long circulating IgG antibodies. As a result, a substantial proportion of the activity administered will have decayed at the time the radiolabeled MAbs have localized in the tumor.

Because of the immunogenicity of the murine form of the MN-14 MAb (seven out of nine patients developed a HAMA-response), the antibody was humanized. To date, three reports have been published on RIT using the humanized MN-14 in patients with advanced CRC. Behr et al.[97,98] and Hajjar et al.[99] tested the toxicity and therapeutic efficacy of ^{131}I-labeled hMN-14 in patients with CRC. Behr et al. focused on patients with small-volume liver metastases (<2 cm) of CRC, whereas Hajjar et al. included patients with gross metastatic disease of colorectal, pancreatic or gastric cancer. Behr et al. found the maximal tolerated activity dose of ^{131}I-hMN-14 at 2220 MBq/m^2, with higher doses leading to grade IV haematological toxicity. Possibly due to a different patient population, Hajjar et al. reported the MTD to be 1480 MBq/m^2. Hajjar et al. did not observe responses, whereas Behr et al. reported three partial remissions and eight minor responses in their larger series of 30 patients.

Interestingly, Behr et al. were the first to report on the administration of adjuvant RIT using ^{131}I-hMN-14 at MTD (2220 MBq/m^2) in nine patients within six weeks after complete resection of liver metastases of colorectal origin.[98] At a median follow-up of 27 months, only two patients had developed a recurrence. Although seemingly promising, these results should be interpreted cautiously, as the group of patients is small and the study noncontrolled. Nevertheless, as mentioned previously, in solid cancers adjuvant RIT should be most effective, since tumor uptake of the radiolabeled MAb and thus the tumor-absorbed radiation dose increases with decreasing tumor size.

Wong et al. published three articles reporting the results of ^{90}Y-based RIT, using the MAb cT84.66 in patients with CRC.[100-102] cT84.66 is a high affinity (Ka=1.16 x 10^{10} M^{-1}) chimeric anti-CEA MAb. After showing effective localization of the radiolabeled MAb in three patients with metastatic CRC,[100] an activity dose escalation study was carried out in a total of twenty-two patients with CEA-positive malignancies, of which 18 were CRCs.[102] The MTD of ^{90}Y-cT84.66 was reached at 814 MBq/m^2, with reversible thrombocytopenia and leukopenia being dose-limiting. Antitumor effects were observed in five patients, including stable disease in three and mixed responses in two.

In an attempt to improve the efficacy of ^{90}Y-cT84.66, it was combined with 5-fluorouracil (5-FU), delivered as a five-day continuous infusion.[101] The MTD of this combination was reached at 618 MBq/m^2 and 1000 mg/m^2/day. Although no objective responses were recorded, eleven patients with previously progressive disease displayed stable disease for three to eight months and one patient showed a mixed response.

Other anti-CEA antibodies that have been tested for radioimmunotherapeutic application include F656 and F023C5.[103] Ychou et al. investigated the toxicity and MTD of single doses of ^{131}I-labeled F(ab')2 fragments, derived from MAb F6, in ten patients with nonresectable liver metastases of colorectal origin, who failed conventional

chemotherapy. The F6 MAb is a murine IgG1 antibody, which recognizes the Gold-1 CEA-specific epitope.[104] Activity doses administered varied between 3219 MBq and 11100 MBq. Haematological toxicity was limited to grade II up to 9250 MBq, whereas after administration of 11100 MBq ^{131}I-F(ab')2 grade IV haematological toxicity developed, which was resolved by means of autologous bone marrow support. Out of nine evaluable patients, one patient showed a partial response of one small liver metastasis, two patients had stable disease, whereas the remaining six showed progression of disease two to three months after therapy.
Behr et al. investigated the therapeutic efficacy of a single administration of ^{131}I-labeled MAb F023C5 in ten patients with small volume metastatic disease of CRC (all lesions ≤ 3 cm).[103] F023C5 is a murine IgG1 MAb with a relatively low affinity constant (0.5×10^7 M^{-1}) for CEA. The administered activity doses varied from 1850 MBq/m^2 to 2960 MBq/m^2, although the MTD had not yet been reached at the highest dose level. Radioimmunoscintigraphy showed that all known tumor localizations were targeted. Dosimetric analysis indicated that the tumor absorbed radiation dose increased exponentially with decreasing tumor size, with the highest dose of 0.50 cGy/MBq observed in a 0.5 cm tumor lesion. One patient had a complete remission, two patients had partial remissions whereas four patients showed stabilization of progressive disease, lasting for longer than 12 months.

TAG-72

TAG-72 was first identified in 1985 as the target antigen of the B72.3 antibody, which had been generated against membrane-enriched fractions of human metastatic breast carcinomas.[105] TAG-72 is a high molecular weight glycoprotein complex (240-400 kDa), which is expressed on 80% of colorectal carcinomas, whereas expression on normal tissues is limited.[106] Besides CRC, various other adenocarcinomas, including gastric, pancreatic, breast, lung, prostate and ovarian cancers express the TAG-72 antigen. Anti-TAG-72 RIT has been studied in five trials involving patients with CRC.[107-111] The first report was published in 1992 by Meredith et al.,[108] who treated twelve patients with metastatic colon cancer with fractionated doses of ^{131}I-labeled chimeric MAb B72.3 (IgG4, affinity constant 2.54×10^9 M^{-1}) at total doses of 1036 MBq/m^2 or 1332 MBq/m^2. Bone marrow suppression was the only significant side effect, which was significantly reduced when the total dose was fractionated. Nine of twelve patients developed an antibody response to the chimeric antibody, altering its kinetics in some patients in subsequent therapy courses. A minor response was observed in one patient.
In the subsequent four trials in which TAG-72 was targeted, the murine MAb CC-49 was used. CC-49 is a second generation anti-TAG-72 MAb and has a six-fold higher affinity (1.62×10^{10} M^{-1}) for the TAG-72 antigen as compared to B72.3. Murray et al.[111] and Divgi and coworkers[107] treated patients with metastatic CRC with ^{131}I-labeled CC-49 with the aim to determine its toxicity and efficacy. Murray et al. treated fifteen patients at 2775 MBq/m^2 and did not observe a response. Divgi and coworkers treated

24 patients with escalating doses of 1665-8436 MBq/m² and reported stabilization of disease in six patients four weeks post-therapy and a minor response in one patient, who received a second administration.
Mulligan and co-authors have been the first to investigate the toxicity and MTD of ^{177}Lu-based RIT using the DOTA-conjugated CC-49 MAb in a small series of patients with advanced adenocarcinoma.[110] Of the three patients described, three had colon cancer, five had breast cancer whereas one patient suffered from lung cancer. Unexpected and unexplained uptake in the bone marrow resulted in grade IV haematological toxicity and limited the maximal tolerated activity dose at only 555 MBq/m². Tumor imaging was observed in all patients, although not all known tumor sites were visualized. Despite the relatively low activity doses administered, two patients experienced stabilization of previously progressive disease. Unfortunately, it was not specified which patients showed stable disease.
Meredith et al.[109] performed the only study investigating the efficacy of dual-antibody RIT using two antibodies, directed at TAG-72 and CEA. This was done in an attempt to overcome heterogeneity of tumor antigen expression. In addition, interferon-α (IFN) was added to enhance the expression of both antigens in the tumor. Fourteen patients with metastatic CRC were treated with anti-CEA murine MAb COL-1 and anti-TAG-72 murine MAb CC-49, both labeled with ^{131}I at a total dose of 2775 MBq/m². Although dual-antibody RIT combined with IFN treatment appeared to result in enhanced tumor targeting and consequently higher radiation doses at tumor sites as compared to historical controls, the estimated tumor absorbed radiation doses varied from four to thirteen Gy, which is too low to result in objective responses. The murine CC-49 MAb was highly immunogenic, since human anti-mouse antibody immune responses were recorded in almost all patients in the above mentioned trials.

A33

The A33 antigen is an extensively studied antigen, which is expressed by the epithelia of the gastrointestinal tract as well as by carcinomas of the colon and rectum.[112,113] A33 is a relatively small antigen (MW 43 kDa), which, because of its high and homogenous expression in 95% of metastatic colorectal carcinomas, has been targeted in two phase I/II RIT trials in patients with CRC.[114,115] In these trials, the A33 antigen was targeted using the radioiodinated homonymous murine IgG2a MAb A33. After having shown scintigraphically and by radioactivity measurements of tissue biopsies that MAb A33 preferentially accumulated in metastases of patients with advanced CRC,[116] Welt et al. conducted two phase I/II trials investigating the MTD and therapeutic efficacy of 125I- and ^{131}I-labeled MAb A33. In their first trial, 23 patients with advanced CRC were treated with escalating activity doses of ^{131}I-labeled A33 (1110-3330 MBq/m²).[114] Three mixed responses were observed, whereas in two patients CEA serum levels decreased after RIT. In their second trial, 21 patients with metastatic CRC were treated with A33 labeled with the Auger-electron emitter 125I (1850-12950

MBq/m²).[115] Besides one mixed response, twelve patients experienced stabilization of previously progressive disease.

Ep-CAM

The Ep-CAM antigen, also known as the 17-1A antigen, is a 40 kDa transmembrane glycoprotein, which is expressed by normal gastrointestinal epithelium and at a higher level by several epithelial cancers, such as lung, breast, prostate, ovarian and colorectal carcinoma. It was described by Göttlinger et al. in 1986, who then called it the epithelial cell surface antigen 17-1A.[117] An important feature of the Ep-CAM antigen is that is not shed into the circulation, so that no complexation with circulating MAbs is to be expected. To date, one phase I/II trial has been reported, investigating the efficacy of anti-Ep-CAM RIT, using escalating activity doses (740-9250 MBq) of 125I-labeled murine IgG2a MAb 17-1A in 28 patients with advanced CRC.[118] Ten patients showed stabilization of disease.

CSA-p

CSA-p can be demonstrated in approximately 60% of colorectal carcinomas and was first identified in 1974 by Pant et al.[119] To date, one phase I trial investigating anti-CSA-p RIT has been published. Sharkey et al. administered escalating activity doses of ^{131}I-labeled IgG (403 – 3633 MBq) or F(ab')2 fragments (311 – 2960 MBq) of the murine Mu-9 MAb to 25 patients with advanced gastrointestinal cancer, among which 21 patients with CRC, with the aim to explore the targeting capabilities of Mu-9 and to assess its pharmacokinetics and immunogenicity.[38] Dosimetric analysis of scintigraphic images revealed a two times higher tumor-absorbed radiation dose per administered MBq for the ^{131}I-Mu-9 IgG as compared to that of ^{131}I-Mu-9 F(ab')2, although the dose absorbed by the normal organs, except for the kidneys, was two- to threefold less for the F(ab')2. The majority of patients developed a HAMA response, although levels in patients treated with F(ab')2 fragments were lower. Although the patients described took part in an ongoing RIT study, tumor responses of anti-CSA-p RIT were not reported.

Ovarian cancer

Ovarian cancer has been targeted in RIT trials using mainly antibodies directed against the MUC-1 antigen and a few other tumor-associated glycoproteins, such as TAG-72 and gp-38.[120] Because of its high propensity to stay confined to the peritoneal cavity until very late in the course of the disease, radiolabeled antibodies have been administered intraperitoneally in most trials. Indeed, in several preclinical and clinical studies it was demonstrated that the intraperitoneal route of administration results in higher uptake of radiolabeled antibodies in tumor as compared to the intravenous route of administration.[121-126] Other authors, however, could not confirm an advantage of intraperitoneal over intravenous administration.[127] It has furthermore

been suggested that intravenous administration may result in a more homogeneous uptake in intraperitoneal tumors, as compared to intraperitoneal administration.[128] Several clinical trials on RIT in patients with ovarian cancer have been published. The best representative trials have been summarized in Table 4.

MUC-1

One of the very first clinical trials of RIT in patients with ovarian cancer was carried out by Epenetos et al., who treated 24 patients with stage III ovarian cancer, after cytoreductive surgery and first-line platinum-based chemotherapy, with escalating activity doses of ^{131}I-labeled MAb HMFG-1, HMFG-2, AUA 1 or H17E2.[129] Clinical responses were limited to those sixteen patients with minimal residual or small-volume disease (<2 cm). Four patiens experienced complete responses for up to 3 years, whereas five patients showed stable disease for up to 20 months. Interestingly, three patients that had microscopic disease only at the time of RIT showed a pathologically confirmed complete response, as demonstrated during a second-look laparoscopy.
In a second trial, Nicholson et al. tested the efficacy of 666 MBq/m^2 ^{90}Y-labeled HMFG-1 MAb in 21 patients with stage IC-IV ovarian cancer who had no macroscopic disease after cytoreductive surgery and completion of platinum-based chemotherapy.[130] Sixteen patients were still alive ten years after treatment, which was significantly better than the median survival of less than four years of a matched historical control group. Epenetos et al. reported later that within the subgroup of 21 patients who experienced a complete remission following cytoreductive surgery, chemotherapy, and intraperitoneal RIT, the median survival had not yet been reched at a follow-up of >12 years.[131] Based on these promising results a multicenter phase III RCT was initiated, comparing the efficacy of ^{90}Y-DOTA-HMFG1 to no treatment in a total of 447 patients with ovarian cancer with no evidence of disease following cytoreductive surgery and platinum- or taxol-based chemotherapy, as confirmed by diagnostic laparoscopy. Unfortunately, preliminary results have indicated that adjuvant RIT did not result in a significant survival benefit in these patients.[132]

TAG-72

The majority of ovarian carcinomas express TAG-72.[133] The first antibody used for radioimmunotherapeutic application was the murine MAb B72.3. After studying the clinical pharmacology, metabolism and tissue distribution and confirming preferential uptake of ^{90}Y-labeled B72.3 in tumor in nine patients with ovarian cancer,[134] Rosenblum et al. performed a dose-escalation phase I study of RIT using ^{90}Y-DTPA-B72.3 in a total of 58 patients with ovarian cancer.[135] Continous intravenous infusion of EDTA was applied in order to suppress the uptake of ^{90}Y in bone, which allowed the administration of higher activity doses. Two minor, and two complete esponses were noted in four patients who received 555-1110 MBq.
Alvarez et al. conducted two phase I/II trials, in which they investigated the toxicity and efficacy of the second-generation anti-TAG-72 MAb CC49, labeled with either ^{90}Y

Table 4. Representative phase I/II radioimmunotherapy trials in patients with ovarian cancer

Reference	Radiopharmaceutical	Target antigen	MAb type	No. of patients	Responses	Special features
Epenetos et al.[129]	^{131}I-HMFG-1, -HMFG-2, ^{131}I-AUA-1, -H17E2	MUC-1 and others	Murine IgG	24	4 CR; 5 SD	
Nicholson et al.[130]	^{131}I-HMFG-1	MUC-1	Murine IgG	21	74% 10-year survival	
Rosenblum et al.[135]	^{90}Y-B72.3	TAG-72	Murine IgG	58	2 CR; 2 MR	EDTA added to reduce bone uptake
Alvarez et al.[136]	^{177}Lu-CC49	TAG-72	Murine IgG	27	1 PR out of 13 patients with measurable disease	
					2 SD out of 9 patients with <1 cm tumors	
					4 disease-free for 6-35 months of 5 patients with microscopic disease	
Meredith et al.[138]	^{177}Lu-CC49	TAG-72	Murine IgG	34	4 PR out of 17 patients with measurable disease	Combined with subcutaneous IFNa-2b and intraperitoneal chemotherapy
					4 progression-free for 18-37 months of 27 patients with microscopic disease	
Alvarez et al.[137]	^{90}Y-CC49	TAG-72	Murine IgG	20	2 PR out of 9 patients with measurable disease	Combined with subcutaneous IFNa-2b and intraperitoneal chemotherapy
					3 disease-free for >18 months of 11 patients with microscopic disease	
Crippa et al.[140]	^{131}I-MOv18	gp-38	Murine IgG	16	5 CR; 6 SD	
Van Zanten-Przybysz et al.[141]	^{131}I-MOv18	gp-38	Chimeric IgG	3	3 SD	

SD, stable disease; MR, minor response; MixR, mixed response; PR, partial response; CR, complete response

or ^{177}Lu.[136,137] In their first trial, 27 patients with ovarian cancer, who failed conventional chemotherapy, were treated with ^{177}Lu-CC49. The maximal tolerated activity dose was established at 1665 GBq/m^2, at which dose-limiting myelosuppression was encountered.[136] Serial radioimmunoscintigraphic imaging revealed intraperitoneal tumors in 19 out of 27 patients up to 19 days after administration of the radiolabeled antibodies. There seemed to be a correlation between tumor mass and response. Of the 13 patients with measurable disease, one showed a partial response. Of the nine patients with <1 cm tumor nodules, two patient showed stable disease for 4 and 5 months, respectively, whereas the remaining 7 patients progressed within 21 months. Five patients had microscopic disease only on study entry. Of these, one showed evidence of progression at 10 months, whereas the remaining four patients remained disease-free at 6-35 months post-treatment.

Meredith et al. subsequently tested the feasibility and efficacy of the combination of intraperitoneal RIT using ^{177}Lu-CC49 and intraperitoneal Paclitaxel (100 mg/m^2) in 34 patients with ovarian cancer.[138] Chemotherapy was given 48 hours prior to the administration of the radiolabeled antibodies. In addition patients received subcutaneous injections of IFN α2b in order to enhance the expression of TAG-72. The MTD of ^{177}Lu-CC49 within this combination treatment regimen was reached at 1480 MBq/m^2, above which dose-limiting bone marrow toxicity occurred. Four out of seventeen patients with measurable disease on CT had partial responses, whereas four out of 27 patients with nonmeasurable disease experienced progression-free time intervals of >18 months.

After showing the feasibilty of the combination of RIT using ^{177}Lu-CC49 and Paclitaxel in a phase I trial, Alvarez et al. treated twenty patients with persistent or recurrent ovarian cancer after conventional cytoreductive surgery and chemotherapy, with a combination of RIT using ^{90}Y-labeled CC49 and chemotherapy using paclitaxel, with the aim to feasibility and MTD of ^{90}Y-CC49 when applied within an intraperitoneal combined modality treatment protocol.[137] Patients received subcutaneous injections of interferon (IFN) alpha2b as well. The MTD of ^{90}Y-CC49 was reached at 895 MBq/m^2 with hematological toxicity being dose-limiting. Of nine patients with measurable disease, two had partial responses for up to 4 months. Eleven patients had nonmeasurable disease. Of these four patients remained disease-free, three of whom longer than 18 months post-treatment.

gp-38

Another tumor-associated glycoprotein targeted with radiolabeled antibodies in ovarian cancer is gp-38, a 38 kDa protein that was identified as the alpha isoform of the folate receptor. Being a marker for malignant transformation in the ovary, it is constitutively expressed at high levels in up to 90% of nonmucinous ovarian carcinoma, while expression levels at other epithelial tissues are low.[139]

Anti-gp-38 RIT has been applied in patients with ovarian cancer, using the murine or chimeric MOv18 MAb. After demonstrating preferential uptake of intraperitoneally

or intravenously administered ^{131}I-labeled murine MOv18 MAb in tumor in 30 patients with ovarian cancer,[126] Crippa et al. tested the efficacy of high-dose intraperitoneal RIT using ^{131}I-MOv18 (3700 MBq) in sixteen patients with minimal or small volume ovarian cancer (macroscopic disease < 5 mm or positive blind biopsies and/or positive peritoneal washing).[140] Clinical follow-up and/or third-look evaluation performed 90 days after RIT showed complete response in five patients, stable disease in six patients and progressive disease in the remaining five patients. Of the five patients that experienced a complete response, one patient remained clinically disease-free for 34 months, whereas the remaining four patients relapsed after a mean disease-free period of 10.5 months. The murine MOv18 MAb, however, was highly immunogenic, with HAMA formation in fifteen out of sixteen patients.
At the Vrije Universiteit medical center (Amsterdam, The Netherlands) Van Zanten-Przybysz et al. studied the pharmacokinetics and efficacy of RIT using ^{131}I-labeled chimeric MAb MOv18 (3 GBq) in three patients with ovarian cancer.[141] In addition, the tumor-absorbed radiation dose was estimated by means of dosimetric analysis of repeated radioimmunoscintigraphic images. All patients experienced stabilization of disease for two to six months, as assessed by CT scans and serum CA 125 measurements. Tumor-absorbed radiation doses varied from 600 to 3800 cGy. None of the patients developed a HACA response.

Breast cancer

Because of its common prevalence, its relative radiosensitivity, and the availability of MAbs against TAAs, breast cancer has been one of the first malignancies targeted with radiolabeled antibodies. TAAs targeted in RIT in patients with breast cancer include CEA, MUC-1, and L6, as recently reviewed by Sally DeNardo.[142] The best representative trials have been summarized in Table 5.

CEA

Although CEA expression has been reported in only 19% of breast cancers,[143] anti-CEA radioimmunodetection and RIT has been evaluated in patients with breast cancer with several MAbs, including the murine IgG1 NP-4, and the second-generation, high-affinity chimeric MAb T84.66. ^{131}I and ^{90}Y were the radionuclides used. RIT using ^{131}I-labeled NP-4 has been investigated in a mixed adenocarcinoma patient group with some responses in patients with breast cancer.[142] Wong et al. evaluated the toxicity of single-dose RIT using ^{90}Y-labeled DTPA-conjugated chimeric MAb T84.66 in patients with metastatic breast cancer and established the MTD at 814 MBq/m^2, with grade 3 reversible myelosuppression being dose-limiting.[144,145] After imaging studies confirmed tumor-targeting, six patient received higher activity doses of ^{90}Y-DTPA-cT84.66 in combination with autologous PBSC support. Clinical responses were achieved, including stabilization of disease progression for up to four months, improvement in bone scans, a 50% reduction of a metastasis, and the reduction of pleu-

Table 5. Representative phase I/II radioimmunotherapy trials in patients with breast cancer

Reference	Radiopharmaceutical	Target antigen	MAb type	No. of patients	Responses	Special features
Wong et al.[144,145]	90Y-T84.66	TAG-72	Chimeric IgG	6	2 SD; 1 PR	Combined with autologous PBSC transplantation
DeNardo et al.[147]	90Y-BrE-3	MUC-1	Murine IgG	6	1 PR; 2 symtom improvement	
Schrier et al.[148]	90Y-BrE-3	MUC-1	Murine IgG	9	4 PR	Combined with autologous PBSC transplantation
Richman et al.[146]	90Y-BrE-3	MUC-1	Humanized IgG	12	2 PR; 2 SD; 4 symptom improvement	Combined with autologous PBSC transplantation
DeNardo et al.[150]	131I-L6	L6	Chimeric IgG	10	3 PR; 1 short-term SD	
Richman et al.[54]	131I-L6	L6	Chimeric IgG	3	1 symptom improvement + decreased serum tumor markers	

SD, stable disease; MR, minor response; MixR, mixed response; PR, partial response; CR, complete response

ral effusion or bone pain for up to 14 and 3 months, respectively, which suggests that stem-cell supported ^{90}Y-DTPA-cT84.66-based RIT might have therapeutic potential in patients with breast cancer.

MUC-1

RIT using antibodies directed against the MUC-1 antigen has been tested in patients with breast cancer using various antibodies, including BrE-3 and the m170.[146] Denardo et al. conducted a phase I dose-escalation trial, with the aim to determine the pharmacokinetics and maximal tolerated dose of ^{90}Y-MX-DTPA-labeled BrE-3 in six patients with metastatic breast cancer.[147] Whereas the highest dose applied was only 342 MBq/m^2, three patients showed objective evidence of a response, e.g. a partial response in one, and reduction of arm swelling and skin lesions in another. In a subsequent phase I trial the feasibility and efficacy of a single high-dose administration of ^{90}Y-DTPA-BrE-3 combined with PBSC support was investigated in nine patients with metastatic breast cancer, who had been heavily pretreated with chemotherapy.[148] Despite PBSC support, grade 4 thrombocytopenia and leucocytopenia was observed in four and two patients, respectively. Four out of eight patients with measurable disease showed objective partial responses.

Because the majority of the patients rapidly HAMA against the murine BrE-3 MAb, a humanized version of BrE-3 was developed. Kramer et al. investigated the pharmacokinetics and biodistribution of ^{111}In-MX-DTPA-labeled hBrE-3 in seven patients with metastatic breast cancer. hBrE-3 proved to have a lower immunogenicity as compared to the murine BrE-3 (only one patient developed a HAHA response) while tumor-targeting properties were preserved. Dosimetric analysis indicated that a radiation dose of 1.9 ± 0.8 cGy/MBq could be delivered to tumor assuming that the biodistribution of ^{90}Y-MX-DTPA-hBrE-2 would have been similar to that of ^{111}In-MX-DTPA-hBrE-2. A phase I dose-escalation study was then initiated, in which patients with metastatic breast cancer received a single administration of high-dose RIT using ^{90}Y-MX-DTPA-hBrE-2, two weeks later followed by reinfusion of G-CSF mobilized PBSCs. Preliminary reports to date indicate that the maximal tolerated activity dose is substantially increased, with two patients having received 1850 MBq/m^2 without encountering dose-limiting toxicity. Two patients showed partial responses, three patients showed stabilization of previously progressive disease, four patients experienced improvement of symptoms, whereas one patient showed progressive disease.[146]

L6

The L6 cell surface antigen is highly expressed on the various carcinomas, including breast, lung, colorectal and ovarian cancer,[149] and as such, has generated interest as a target for RIT. DeNardo et al. tested the efficacy of anti-L6 RIT using repeated administrations (four monthly cycles) of ^{131}I-labeled chimeric MAb L6 in ten patients with metastatic breast cancer, who had failed conventional chemotherapy.[150] Activity

doses varied from 740-2590 MBq/m² and were administered after a diagnostic imaging dose of ^{131}I-cL6. Myelotoxicity was dose-limiting. Six patients experienced clinically measurable tumor responses, consisting of partial responses in three patients and short-term stabilization of rapidly progressive disease in one patient.
As mentioned previously, Richman et al. reported on three additional patients who were treated with ^{131}I-L6 at 5550 MBq/m² with PBSC support, which controlled bone marrow toxicity.[54] One of these patients, treated with cyclosporin A to prevent HACA formation, received even three therapy cycles, rendering her clinical condition markedly improved for up to nine months.

Renal cell cancer

Several tumor-associated antigens have been targeted in experimental models of RIT of renal cell cancer (RCC). The only antigen used in clinical RIT is G250, which has been targeted using the homonymous MAb G250. Table 6 summarizes the characteristics of the reported trials on RIT using G250 in patients with RCC.
At the Radboud University Nijmegen Medical Center as well as the Memorial Sloan Kettering Cancer Institute several clinical studies have been conducted on the feasibility, toxicity and efficacy of RIT using radiolabeled G250. Oosterwijk et al.[151] determined the optimal protein dose of the murine G250 MAb in fifteen patients with their renal cell cancers still in situ. Seven or eight days prior to tumor nephrectomy, fifteen patients received a diagnostic dose of ^{131}I-labeled G250. Clear tumor localization was observed in all twelve patients with G250 positive tumors and in one of three patients with G250 negative tumors. The smallest lesion visualized was 8 mm, whereas uptake of the radiolabel in the tumor reached as high as 0.12% ID/g.
In a subsequent therapy study, Divgi et al. administered escalating activity doses (1110-3330 MBq/m²) of ^{131}I-labeled G250 to patients with metastatic renal cell cancer.[152] The MTD was reached at 3330 MBq/m², with bone marrow toxicity being dose-limiting. Altough there were no objective responses, seventeen out of thirty-three evaluable patients showed stabilization of disease-progression. The murine G250 MAb, however, proved highly immunogenic since all patients developed a HAMA response.
After the development of the chimeric form of G250, Steffens et al.[153] performed a tumor-targeting study with tracer-doses of ^{131}I-labeled chimeric G250 in patients with renal cell cancer. It was shown that focal uptake of ^{131}I-cG250 in RCC lesions could be very high (up to 0.52% of the injected dose per gram). Dosimetric analysis confirmed that potentially tumoricidal radiation doses, up to 1.95 cGy/MBq, could be guided to RCC lesions.In a subsequent phase I study involving twelve patients with metastatic RCC, Steffens et al. subsequently established the maximal tolerated activity dose of ^{131}I-cG250 at 2220 MBq/m².[154] In this study, one patient showed a partial response, whereas another patient experienced stabilization of previously progressive disease for >6 months.

Table 6. Representative phase I/II radioimmunotherapy trials in patients with renal cell cancer

Reference	Radiopharmaceutical	Target antigen	MAb type	No. of patients	Responses	Special features
Divgi et al.[151]	131I-G250	G250	Murine IgG	33	17 SD	
Steffens et al.[153]	131I-G250	G250	Chimeric IgG	12	1 PR; 1 SD	
Brouwers et al.[154]	131I-G250	G250	Chimeric IgG	27	4 SD out of 15 evaluable patients receiving two courses of RIT	Fractionated RIT
Divgi et al.[155]	131I-G250	G250	Chimeric IgG	15	7 SD	Fractionated RIT

SD, stable disease; MR, minor response; MixR, mixed response; PR, partial response; CR, complete response

After Steffens et al. demonstrated the feasibility of RIT using ^{131}I-labeled cG250 in patients with metastatic renal cell cancer, Brouwers et al. performed a phase I/II trial with the aim to define the feasibility and efficacy of repeated high-dose ^{131}I-cG250.[155] Twenty-seven patients with progressive metastatic renal cell cancer received a therapeutic infusion of 2220 MBq/m^2 ^{131}I-cG250 (=MTD). Three patients experienced grade 4 hematological toxicity after the first therapy course and were considered not eligible to receive a second course of RIT. After completion of the first RIT course, nineteen patients received a second therapeutic infusion of ^{131}I-cG250. Grade 4 hematological toxicity limited the maximal tolerated dose of a second RIT course of ^{131}I-cG250 to 1665 MBq/m^2, or approximately 75% of the MTD of the first RIT course. Dosimetric analysis revealed an inverse correlation between tumor size and radiation absorbed dose, although there was a wide variation in the tumor radiation doses. No objective responses were recorded. Still, four out of fifteen evaluable patients who received two therapy courses experienced stabilization of previously progressive disease for up to six months.

Divgi et al. tested the tolerability and efficacy of fractionated RIT which consisted of up to three therapy courses of ^{131}I-cG250 in fifteen patients with metastatic and progressive renal cell cancer.[156] Thus, it was reckoned, bone marrow toxicity might be reduced, enabling the delivery of higher tumor-absorbed doses. The administered activity dose of the first administration amounted 1110 MBq (30 mCi). In the following administrations, each separated by 2-3 days, the activity dose was determined by "topping up" the whole body activity to 1110 MBq (30 mCi). Five patients received two cycles of therapy, while only two patients were able to complete three cycles. Out of fourteen evaluable patients, seven patients showed stable disease, for 2-11 months. Hematological toxicity was dose-limiting. Furthermore, dosimetric analysis could not substantiate evidence of bone marrow sparing by fractionation.

Brouwers et al. performed an intra-patient analysis comparing the targeting properties of ^{111}In-labeled ITC-DTPA-cG250 with ^{131}I-labeled cG250 in five patients with metastatic RCC.[157] Four days post-injection of the radiolabeled antibodies, ^{111}In-ITC-DTPA-cG250 revealed significantly more RCC lesion than ^{131}I-cG250 (47 versus 30). Quantitative analysis of the images furthermore revealed higher activities of ^{111}In-ITC-DTPA-cG250 than ^{131}I-cG250 in 20 of 25 lesions. Most likely, the higher activities of ^{111}In-ITC-DTPA-cG250 in the metastatic lesions are caused by internalization and subsequent intracellular retention of the radiolabel, which implies that residualizing radionuclides, such as ^{90}Y or ^{177}Lu, might be better suitable for RIT using cG250 in patients with RCC than ^{131}I. Currently, at the Radboud University Nijmegen Medical Center a phase I/II therapy trial has been initiated, investigating the efficacy of ^{177}Lu-labeled ITC-DTPA-cG250 in patients with RCC.

Pretargeted RIT trials

Preclinical and clinical research on the application of pretargeted RIT using the (strept)avidin-biotin or bispecific antibody methodology has been extensively reviewed by Boerman et al.[41] To date, four reports have been published on pretargeted RIT using therapeutic activity doses in patients with metastatic carcinoma. Table 7 summarizes the characteristics of the reported trials on pretargeted RIT in patients with epithelial cancers.

Bardies and colleaugues investigated the feasibility of pretargeted RIT in five patients with metastatic medullary thyroid cancer and five patients with metastatic small-cell lung cancer, whose tumors were pretargeted with the anti-CEA x anti-DTPA-In bsMAb F6-734.[158] Four days later, patients received a diagnostic dose (222-370 MBq) ^{131}I-labeled di-DTPA. Dosimetric analysis of radioimmunoscintigraphic images revealed that the radiation dose to the medullary thyroid carcinoma lesions was much higher (range: 113 - 470 Gy/MBq) than the radiation dose to the SCLC lesions (range: 4.6 - 22 Gy/MBq), suggesting that MTC might be a more suitable target for this pretargeted RIT. Subsequently, Kraeber-Brodéré et al. tested the feasibility and therapeutic efficacy of pretargeted RIT in 26 patients with recurrent medullary thyroid carcinoma.[159] Tumors were pretargeted with 20-50 mg anti-CEA x anti-DTPA-In bsMAb, after which 1.48 - 3.7 GBq ^{131}I-di-DTPA was administered four days later. Dose limiting toxicity was hematological; an activity dose of 1.78 GBq/m^2 could be administered safely. The radiation dose to the tumor ranged from 7.9 - 500 Gy/MBq. Of the seventeen evaluable patients, five showed minor responses, four experienced symptom improvement (pain relief), whereas in four patients serum calcitonin levels decreased.

As expected, in a similar pretargeted RIT study reported by Vuilez et al. in fourteen patients with small cell lung cancer, the radiation doses to the tumor were lower 7.0-87 Gy/MBq.[160] In that study, patients received a total of 1.48-6.66 GBq ^{131}I-diDTPA four days after pretargeting with 20-80 mg anti-CEA x anti-DTPA-In bsMAb. Interestingly, the MTD of the ^{131}I-labeled di-DTPA peptide in these patients was much higher (5550 MBq) than the MTD in patients with medullary thyroid cancer, possibly because most patients with disseminated medullary thyroid cancer have micrometastatic disease in the bone marrow. In the latter study in patients with small cell lung cancer, the activity dose was further escalated, until second organ toxicity. Patients receiving 5550 MBq or more, PBSCs were harvested prior to treatment and reinfused 10-15 days after injection of the radioactivity. Out of 12 evaluable patients, two patients showed a partial response.

In a phase I trial Kraeber-Brodéré et al.[161] attempted to optimize pretargeted RIT with respect to the bispecific hMN-14xm734 MAb dose and the time interval between the administration of the bispecific MAb and a ^{131}I-labeled peptide. Thirty-five patients with CEA-expressing cancers were included, among whom 15 patients with CRC. Optimization of the BsMAb dose and the time interval between both injections allowed

Table 7. Pretargeted radioimmunotherapy trials in patients with epithelial cancers

Reference	Radiopharmaceutical	Target antigen	MAb type	No. of patients	Responses	Special features
Kraeber-Brodere et al.[158]	F6 x 734 + 131I-diDTPA	CEA	Murine	26	4 MR; 4 symptom improvement; 4 reduction serum calcitonin levels	
Vuillez et al.[159]	F6 x 734 + 131I-diDTPA	CEA	Murine	14	2 PR out of 12 evaluable patients	Combined with autologous PBSC transplantation
Kraeber-Brodere et al.[160]	hMN-14 x m734 + 131I-hapten	CEA	Humanized IgG1	35	Not reported	
Knox et al.[161]	NR-LU-10/SA + 90Y-Biotin	Ep-CAM	Murine IgG	25	2 PR; 4 SD	

SD, stable disease; MR, minor response; MixR, mixed response; PR, partial response; CR, complete response

the administration of high activity doses, that, based on dosimetric analyses, could be tumoricidal. Antitumor effects, however, were not reported.
Knox et al.[162] performed a phase II trial investigating the therapeutic efficacy of pretargeted RIT in 25 patients with metastatic colon cancer, using the anti-Ep-Cam MAb NR-LU-10, conjugated to Streptavidin (NR-LU-10/SA) and ^{90}Y-DOTA conjugated biotin. One day after the administration a clearing agent (biotin-galactose-albumin) was administered to remove unbound circulating NR-LU-10/SA. Finally, one day after administration of the clearing agent, ^{90}Y-DOTA-conjugated biotin was administered. Due to reactivity of NR-LU-10/SA with bowel epithelium as well as the rapid renal clearance of ^{90}Y-DOTA-biotin from the blood, haematological toxicity was less severe than nonhaematologic toxicity, which consisted of grade 3, 4, or even 5 diarrhoea in 32% of patients. Two patients, however, experienced partial responses, whereas four patients had stabilization of progressive disease for up to 20 weeks. Importantly, two patients showed significant elevations of serum creatinine levels seven and eight months after therapy, that could not be explained by disease progression. This important observation once again points at the potential danger of the application of radiolabeled haptens in pretargeted RIT, with respect to irreversible radiation damage to the kidneys.

Conclusion

The appealing concept of systemically delivering targeted radiotherapy by means of radiolabeled antibodies for the treatment of cancer has had a history of more than 50 years. With the introduction of MAbs thirty years ago, a great number of tumor-associated antigens have been identified. Since then, huge progress has been made in understanding the complexity and difficulties of this treatment modality. To date, RIT has emerged as an accepted treatment for patients with NHL, for which two radiolabeled MAbs have been approved in the US by the FDA for therapeutic application. The therapeutic application of radiolabeled MAbs in solid cancers, however, has met considerable difficulties, mainly because uptake of the radiolabeled MAbs in tumor tissue in many cases is too low to result in tumoricidal radiation doses to the tumors. The limited number of clinical phase I/II RIT trials, using different antibodies and radionuclides at various activity doses yet precludes the drawing of any firm conclusion on the potential promise of RIT in the treatment of patients with solid cancers. Most patients included in these trials suffered from advanced, mostly bulky, metastatic disease, which is a highly unfavorable setting for the application of radiolabeled antibodies. As a result, radiation doses delivered to the tumors by the radiolabeled antibodies were probably below tumoricidal doses in most cases. Whereas in the majority of patients in most studies disease progression was observed, a few patients, however, still experienced objective partial remissions or short-term stabilization of previously progressive disease. The observation of some tumor responses in these

heavily pretreated patients with bulky disease once again suggests that RIT in patients with small volume disease may be much more effective. The modest therapeutic results of RIT in patients with solid cancers may therefore leave room for qualified optimism. Although several strategies aimed at increasing the therapeutic window are currently under investigation, patient selection may be the most important factor determining the eventual success of Paul Ehrlich's concept of magic bullets in RIT for patients with solid cancers. Based on the results thus far achieved, the time may have come to design clinical trials in which RIT is added to standard regimens, in order to establish the place of this treatment modality. Since small volume disease has been recognized as the most suitable setting for the application of radiolabeled MAbs in solid cancers, RIT given as adjuvant treatment may prove its true value in patients with solid cancers, such as after resection of primary tumors with a high risk of distant metastases.

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2

Peritoneal carcinomatosis of colorectal origin; incidence and current treatment strategies

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Besides the lymphatic and hematogenous routes of dissemination, colorectal cancer (CRC) frequently gives rise to transcoelomic spread in the peritoneal cavity, which, ultimately, may cause peritoneal carcinomatosis (PC). During the course of disease of a patient with a gastrointestinal cancer, it is believed that there are two time points at which intraperitoneal spread or seeding of cancerous tumor emboli may occur.[1] Firstly, intraperitoneal spread may occur pre-operatively as a result of full-thickness invasion of the bowel wall by an invasive cancer. Pre-operative seeding may also occur as a result of the rupture of a structure by a non-invasive tumor, such as the mucus producing cystadenocarcinoma of the appendix. This leads to the clinical syndrome of pseudomyxoma peritonei, characterized by massive intraperitoneal accumulation of mucus in the absence of organ metastases. This rare clinical entity has been reviewed elsewhere and is beyond the scope of this paper.[2,3] Secondly, intraperitoneal spread may be induced iatrogenically during surgery, when in-transit tumor cells or emboli escape from dissected lymph vessels, the bowel lumen or reach the peritoneal cavity through blood spill from the surgical field.

Until recently, most oncologists considered PC a terminal condition, only to be palliated with systemic chemotherapy. However, it has been estimated that in approximately 25% of patients with recurrent CRC, the peritoneal cavity seems to be the only site of metastatic disease, even after a detailed diagnostic work-up of the liver and lungs.[4,5] Because of this observation some surgical oncologists hypothesized that, similar to liver metastases, PC may be a first step of dissemination and should, therefore, not necessarily be interpreted as generalized disease.[6,7] In recent years, a new therapeutic approach has been investigated in a dozen medical centers worldwide, aimed at locoregional control and long-term survival.[8] This approach is based on aggressive cytoreductive surgery after which the peritoneal cavity is perfused with chemotherapeutics, sometimes under hyperthermic conditions, in order to sterilize (minimal) residual disease.

Here, the available literature on the incidence and prognostic significance of microscopic peritoneal dissemination of tumor cells as well as on the incidence of true intraperitoneal recurrence in CRC is reviewed. Finally, the results of clinical trials investigating the feasibility and efficacy of cytoreductive surgery followed by intraperitoneal chemotherapy (IPEC) for the treatment of PC of colorectal origin are discussed.

Incidence and prognostic significance of intraperitoneally seeded tumor cells in colorectal cancer

Between 1980 and 2004, twelve papers have been published reporting the results of clinical studies on the incidence and/or prognostic relevance of microscopic peritoneal dissemination of tumor cells in patients undergoing curative surgery for primary CRC.[9-20] These trials focussed on patients with primary colorectal cancer who underwent surgery with curative intent and excluded patients with evidence of hematoge-

nous metastases, PC or ascites or patients who had undergone emergency surgery for obstructive of perforated cancers. The relevant trial characteristics are summarized in Table 1.

Although the inclusion and exclusion criteria were similar, the studies differed with respect to design, methods of collecting the specimens and pathological staining techniques. Four trials explicitly mentioned the inclusion of consecutive patients.[12,15,17,19] In eight trials material for cytological analysis was harvested by pouring normal saline onto the serosal surface of the tumor bed immediately after entering the abdominal cavity and, in some studies, again after the resection prior to closure of the abdomen.[9-13,17-19] In two trials, material was obtained by gently touching, scraping or brushing the serosa overlying the tumor mass using a slide glass or brush,[15,20] whereas in two trials both aforementioned techniques were used.[14,16] In all but one trial an attempt was made to identify cancer cells using conventional cytology and/or immunocytochemistry. One trial used the sensitive polymerase-chain reaction (PCR) technology to detect the presence of free cancer cells in the peritoneal cavity.[10]

In three trials an attempt was made to determine the sensitivity and/or specificity of the used method by searching for tumor cells in peritoneal lavage fluid obtained from patients with well-known peritoneal metastases and/or benign diseases undergoing surgery.[9,10,17] In two of the three trials specificity was 100%,[10,17] whereas in the third trial nonspecific immunocytochemical staining in one of six control patients with diverticular disease resulted in a specificity of 83%.[9] One trial included a well-defined positive control group of 22 patients with well-established PC, nineteen of whom had tumor-positive cytology, corresponding to a "sensitivity" of 86%.[17]

Incidence of peritoneal seeding

Peritoneal lavage fluid obtained prior to resection proved tumor positive in 3% to 28%,[9-14,16-20] whereas scraping the serosa overlying the primary tumor site or pressing slide glasses on the serosa overlying the tumor generally resulted in somewhat higher incidence rates of 15% to 42%.[14,16,20] Interestingly, Uras et al.[16] and Ojima et al.[14] tested the accuracy of serosal stamp cytology by including serosal stamps of bowel surfaces far away from the primary tumor. These control stamps were tumor-positive in 3%.

In four trials, peritoneal lavage was performed prior to as well as after resection of the tumor. Where reported, the conversion rate from tumor-negative pre-resection to tumor-positive post-resection lavage fluid, apparently as a result of the surgery, varied considerably from 0 till 10%.[10,11]

Prognostic significance of peritoneal seeding

Several authors have made an attempt to (univariately) correlate the finding of tumor-positive cytology with various clinicopathological parameters, including pathological TNM stage, malignancy grade, locoregional recurrence rate and/or survival. Although

Table 1. Incidence of exfoliated tumor cells in the peritoneal cavity during surgery in patients with colorectal cancer

Reference	No. of patients	Acquisition method	Detection method	Positive prior to tumor handling	Positive prior to closure	Conversion rate
Zeng et al.[20]	65	Serosa	Conv	23%	NA	NA
Solomon et al.[15]	103	Serosa	Conv	NA	15%	NA
Uras et al.[16]	72	PL; Serosa	Not reported	PL 14%; Serosa 42%	NA	NA
Horattas et al.[11]	50	PL	Conv	10%	10%	0%
Kim et al.[13]	38	PL	Conv/ICC	Not clear	Not clear	Not clear
Wind et al.[17]	88	PL	Conv	28%	NA	NA
Guller et al.[10]	39	PL	PCR	15%	23%	10%
Yamamoto et al.[18]	189	PL	Conv	6%	NA	NA
Ojima et al.[14]	34	PL; Serosa	Conv	PL 3%; Serosa 29%	NA	NA
Bosch et al.[9]	53	PL	Conv/ICC	17%	13%	not clear
Kanellos et al.[12]	113	PL	Conv	20%	NA	NA
Yang et al.[19]	143	PL	Conv/ICC	3%	NA	NA

PL peritoneal lavage; Conv conventional biochemical staining; ICC immunocytochemistry; NA not applicable

in ten trials the incidence of tumor-positive cytology correlated significantly with more advanced stages of disease, especially T-stage,[11,12,16,17,19,20] Solomon et al.[15] could not confirm this correlation in a large prospective series of 105 patients with CRC. Instead, Solomon et al. found tumor cells on the serosal surface of resection specimens more frequently after resections of distal tumors, that required anterior or abdominoperineal resections.
Interestingly, Yamamoto et al.[18] and Kanellos et al.[12] found tumor-positive cytology to be significantly correlated with the risk of intraperitoneal recurrence. Both groups used similar techniques of instilling normal saline in the peritoneal cavity immediately after entering the abdomen in 113 and 189 patients undergoing curative resection for CRC, respectively. Conventional Papanicolaou and Giemsa staining of the peritoneal lavage revealed tumor cells in 5.8% and 20% of patients, respectively. The relative risk of intraperitoneal recurrence in those patients with tumor-positive cytology as opposed to patients with tumor-negative cytology was 16.5 (95% CI 4.8-57.5, P=0.0004) and 2.9 (95% CI 1.0-8.2, P=0.047), respectively.
Two trials, finally, reported that tumor-positive cytology correlated with impaired overall or disease-free survival.[10,17] However, in none of the studies multivariate analyses were carried out to determine whether tumor-positive cytology has an independent prognostic impact or is merely a confounding prognostic indicator.

Incidence of intraperitoneal recurrence in colorectal cancer

Since hematogenous metastases have always been the main cause of disease-related death in CRC, the intraperitoneal route of dissemination has long been regarded as less important. In the nineteen-seventies and -eighties, however, several authors published retrospective studies concerning the patterns of failure in patients with CRC, recognizing the peritoneal cavity as a common site of recurrence after potentially curative surgery. These patient series were either retrospective clinical follow-up studies,[21-28] reoperation series,[29,30] or autopsy studies of patients who had succumbed to CRC.[23,31] Table 2 summarizes the incidence rates of intraperitoneal failure reported in these studies. It should be noted that since in the late nineteen-eighties the surgical technique of resection in rectal cancer has changed towards total mesorectal excision (TME) and preoperative radiotherapy, the local recurrence rates after surgery for rectal cancer has dramatically decreased to less than 3%.[32] Whether or not the introduction of the TME technique has affected the incidence of PC is unknown.
Clinically evident locoregional failure, in most studies defined as recurrence in the bowel anastomosis or in the resection bed was reported in 5%-18% after curative resection of colon cancer. PC was reported in 4%-12% after curative resection of colon cancer, and in 2%-19% after curative resection of rectal cancer. Recently, Jayne et al.[33] retrospectively analyzed a large series of 3019 patients with CRC: 214 patients (7%) had synchronous PC at the time of resection of the primary tumor, whereas another

Table 2. Incidence of local recurrence or peritoneal carcinomatosis after resection of colorectal cancer

Reference	Total No.of patients	No. colon cancer	No. rectal cancer	% LR total	% LR colon cancer	% PC total	% PC colon cancer	% PC rectal cancer
Clinical series								
Malcolm et al.[24]	285	217	68	3.9%	5%	13%	12%	19%
Cass et al.[21]	280	129	151	23%	18%	28%	8%	2%
Russell et al.[28]	94	94	0	7%	7%	12%	12%	NA
Mendenhall et al.[25]	140	0	140	29%	NA	3%	NA	3%
Olson et al.[27]	281	214	67	9%	7%	_	_	_
Minsky et al.[26]	294	294	0	9%	9%	4%	4%	NA
Gilbert et al.[23]	31	25	6	36%	_	3%	_	_
Jayne et al.[33]	2756	1289	1467	_	_	4.9%	4.8%	5.0%
Reoperation series								
Gunderson et al.[29]	91	91	0	48%	48%	21%	21%	NA
Tong et al.[30]	64	64	0	48%	48%	44%	44%	_
Autopsy series								
Russell et al.[31]	53	53	0	38%	38%	36%	36%	NA
Gilbert et al.[23]	45	25	20	67%	_	40%	_	_

LR local recurrence; PC peritoneal carcinomatosis

135 patients (4.5%) developed metachronous carcinomatosis. Of the patients with synchronous PC, 58% did not seem to have systemic metastatic disease.
Gunderson et al.[29] studied the areas of failure in 91 patients with extrapelvic Dukes B or C colon cancer who underwent a planned second-look laparotomy six to twelve months after a potentially curative resection. Locoregional recurrence either alone or as a component of failure was confirmed in 48% of patients. PC as the sole pattern of recurrence or as a component of failure was found in 4% and 21%, respectively. Tong et al.[30] mapped the sites of failure in patients who required relaparotomy for suspected recurrent proximal colon cancer and found local recurrence in 47% and diffuse PC in 44% of patients.
Gilbert et al.[23] showed that in a autopsy series of 45 patients who had succumbed to CRC, 18 patients had PC. In a similar autopsy series of 53 patients who died of colon cancer, Russell et al.[31] reported a local recurrence rate of 38%, whereas 36% of patients had PC.
These data indicate that in patients with CRC, intraperitoneal recurrence is a rather common phenomenon with important clinical consequences for both medical and surgical oncologists.

Systemic chemotherapy for peritoneal carcinomatosis of colorectal origin

To date, four clinical studies have been published dedicated to the efficacy of chemotherapeutic treatment of patients with PC of colorectal origin. In 1989, Chu et al.[34] prospectively analyzed a series of 100 patients with PC of nongynecologic cancers, among whom 45 patients with CRC, with the aim to identify prognostic factors. In those patients with CRC, of whom the majority was treated with 5-FU and Leucovorin (LV), median survival was six months. Shorter disease-free interval, the presence of lung metastases, and the presence of ascites correlated significantly with decreased survival. In 2000, Sadeghi et al.[35] reported the results of a French prospective multicenter study of 370 patients with PC of nongynecologic malignancy. The 118 patients with PC of colorectal origin in this study had a median survival of only 5.2 months. Jayne et al.[33] recently published an extensive series of 3019 patients with CRC. The 392 patients who presented with PC had a median survival of 7 months. Finally, Verwaal et al.[36] conducted a phase III randomized controlled trial, comparing ultraradical cytoreductive surgery and adjuvant hyperthermic intraperitoneal chemotherapy (HIPEC) and systemic 5-FU-based chemotherapy with systemic chemotherapy and palliative surgery in patients with PC of colorectal or appendiceal origin, but without evidence of extraperitoneal, metastatic disease. Overall median survival of the 50 patients who were treated with systemic chemotherapy and palliative surgery was 12.6 months, with a two-year survival rate of approximately 18%. Median time to disease progression was 7.6 months. The better survival of the patients that received chemo-

therapy and conventional surgery within this RCT as compared to the above mentioned results reported by other authors, is probably due to patient selection, since patients had to be medically fit to undergo cytoreductive surgery and HIPEC and were not allowed to have hematogenous metastases.
Thus, the reported median survival of patients with PC of colorectal origin, with of without hematogenous metastases, after chemotherapy-based treatment, varies between 5.2 and 12.6 months.

Historical perspective of cytoreductive surgery

As mentioned above, the presence of peritoneal metastases has for long been regarded as a lethal disease, characterized by 'contamination' of the entire abdomen, for which complete Ro resection was considered not feasible and, consequently, any attempt thereto futile. In the nineteen-thirties Meigs, an American gynecologist, was the first to advocate cytoreductive surgery followed by adjuvant radiotherapy in patients with ovarian cancer, a disease with a very high propensity to disseminate to the peritoneum.[37] The survival rate after cytoreductive surgery for the treatment of ovarian cancer, however, remained poor. As a result, treatment of ovarian cancer mainly depended on chemotherapy and surgical strategies were not optimized until the late sixties and seventies, when Munnell and Griffiths independently demonstrated that better survival rates could be achieved by more extensive surgery with the size of residual disease being the most important prognostic factor.[38-40] Thus, the concept of ultraradical cytoreduction of PC was introduced.
In view of the improved results of ultraradical cytoreductive surgery in ovarian cancer, surgical oncologists regained a renewed interest in the subgroup of patients with colorectal carcinomatosis without evidence of hematogenous metastases. In 1979, after testing the technique of hyperthermic peritoneal perfusion in fifteen dogs, Spratt et al. were the first to report the results of cytoreductive surgery followed by hyperthermic IPEC using thiothepa in a 35-year-old patient with pseudomyxoma peritonei.[41,42] Except for minor pulmonary atelectasis with bacteremia the patient's postoperative course was uneventful. In the early eighties, this approach was adopted and optimized by Sugarbaker, who investigated its therapeutic efficacy in patients with peritoneal metastases of various gastrointestinal cancers.[43,44] Since then, cytoreductive surgery and adjuvant (hyperthermic) IPEC has been further developed and applied by 30 medical centers worldwide in patients with various kinds of peritoneal surface malignancy, including PC, sarcomatosis and peritoneal mesothelioma.[8] Several reviews have been published describing the techniques of cytoreductive surgery,[45] the rationale of hyperthermia[46] and the various methods of the intraperitoneal administration of chemotherapy.[47]

Cytoreductive surgery and (hyperthermic) intraperitoneal chemotherapy in peritoneal carcinomatosis of colorectal origin

To date, 20 papers have been published reporting the toxicity, complications and survival results of trials investigating the morbidity, mortality and therapeutic efficacy of surgical cytoreduction followed by IPEC, either with or without hyperthermia, in patients with PC of colorectal origin.[36,48-66] Relevant trial characteristics, as summarized in Table 3, differed with respect to design, patient selection and the treatment protocol. Thirteen studies were nonrandomized, single-arm, retrospective patient series, whereas three studies were comparative trials, two of which were randomized.[36,50]

In nine trials only patients with PC of colorectal origin were included,[48,51,52,54-57,59,65] whereas in the remaining six trials patients with PC of appendiceal origin were also included.[49,50,58,64,67,68] Since in the latter trials, the results were analyzed for patients with colorectal carcinomatosis separately, these reports were included in this review. Furthermore, in seven trials patients with hematogenous metastases were eligible for inclusion.[48,50,51,57,58,65,66] IPEC was administered intraoperatively in six trials,[36,51,55-57,64] early postoperatively in four trials,[49,50,54,59] both intraoperatively and early postoperatively in two trials,[53,65] whereas in four patient series various protocols of administering chemotherapy intraperitoneally were utilized.[48,58,66,67] In ten trials, IPEC was given under hyperthermic conditions,[36,48,51,53,55-57,64-67] whereas in five trials it was not.[49,50,54,58,59] Mitomycine-C was the most frequently used cytostatic agent, either alone,[36,56,57,64] or in combination with 5-fluorouracil (5-FU)[48,50,53,58,59,65,67] or Cisplatin.[55] Two trials, finally, used 5-FU alone,[49,54] whereas in one trial oxaliplatin was administered.[51]

The clinical outcomes with respect to long-term survival varied considerably. In short, median survival varied from 12 till 32 months. One-year, 2-year, 3-year and, when reported, 5-year survival rates varied from 65-90%, 25-60%, 18-47% and 17-30%, respectively.

Toxicity and complications of cytoreductive surgery and (hyperthermic) intraperitoneal chemotherapy

Cytoreductive surgery followed by (hyperthermic) IPEC carries a high morbidity and a substantial mortality rate. Postoperative grade III and IV toxicity and complications after cytoreductive surgery and adjuvant IPEC varied from 14% to 55%, whereas treatment-related mortality varied from 0 to 19%.

Morbidity of cytoreductive surgery and (hyperthermic) IPEC can be categorized as surgery- or chemotherapy-related. Five studies have specifically addressed the toxicity and complications related to cytoreductive surgery and (hyperthermic) IPEC, two of which in patients with colorectal or appendiceal cancer only.[53,63,69-71]

Esquivel et al.[69] reported on the complication rate associated with cytoreductive surgery and early postoperative IPEC using mitomycin-C (MMC) and 5-FU in 44 patients with PC of appendiceal, colonic, small bowel or fallopian tube origin. Twenty-two patients had been treated with induction IPEC prior to cytoreductive surgery and postoperative IPEC. The median duration of postoperative ileus was 21 days, which was related to age and the extensiveness of the surgical cytoreduction. In four patients, postoperative hemorrhage required surgical reintervention, whereas two patients developed pneumonia and respiratory failure requiring orotracheal intubation. Enteric complications, including small bowel fistulas, anastomotic disruption, bile leak and pancreatitis occurred in seven patients (16%), of whom six had been treated with induction IPEC. The authors concluded that, since induction IPEC carries an increased risk for postoperative enteric complications, this treatment modality should only be reserved for patients with small volume disease.
Jacquet et al.[53] reported the morbidity and mortality rate of cytoreductive surgery and (hyperthermic) IPEC (MMC) in 60 patients with PC of colorectal or appendiceal origin. Serious complications were encountered in 35% of patients, with anastomotic leakage or bowel perforations being the most frequent and significantly correlated to the number of peritonectomy procedures and the duration of the operation. Three patients (5%) died from treatment-related causes.
Verwaal et al.[63] reported the toxicity of cytoreductive surgery and (hyperthermic) IPEC (MMC) in a series of 102 patients with PC of colorectal or appendiceal origin that had been treated according to the same protocol in prospective phase I, II or III trials. Grade 3, 4, or 5 toxicity, as recorded in accordance to the Common Toxicity Criteria published by the National Cancer Institute (NIH CTC), was observed in 65%. Surgical failures were encountered in 35 patients (35%), resulting in abdominal sepsis in sixteen patients (16%). Twenty-one patients (21%) developed intra-abdominal abscesses that could be drained percutaneously. A total of eight patients (8%) died due to treatment-related causes, six of whom due to abdominal sepsis. Patients with higher tumor loads, incomplete cytoreductions, blood loss exceeding 6 liters and patients with three or more anastomoses had an increased risk of having a complicated postoperative course.
Stephens et al.[71] described a series of 183 patients with PC of gastrointestinal origin who underwent 200 cytoreductive surgeries followed by (hyperthermic) IPEC (MMC). Combined grade 3 or 4 toxicity was noted in 27% of patients, peripancreatitis being the most frequent complication in 6%, followed by fistulization (4.5%), hemorrhage (4.5%) and hematologic toxicity (4%). Three patients (1.5%) died of treatment-related toxicity, two of whom due to severe hematologic toxicity. Treatment-related mortality was 1.5%. The duration of the surgery, the number of peritonectomy procedures, resections of anastomoses were significantly associated with the occurrence of grade 3, 4 or 5 toxicity, whereas (hyperthermic) IPEC-related variables were not.

Table 3. Characteristics of trials investigating the efficacy of cytoreductive surgery and intraperitoneal chemotherapy in patients with peritoneal carcinomatosis of colorectal origin

Reference	Total No. of patients	Origin tumor			I.p. cytostatic agent used	Timing i.p. chemo-therapy
		Colon	Rectum	Appendix		
Sugarbaker et al.[58]	181	Not spec.	Not spec.	130	MMC + 5-FU	Various
Sugarbaker et al.[59]	64	64	0	0	MMC + 5-FU	Early postop.
Schneebaum et al.[56]	15	Not spec.	Not spec.	Not spec.	MMC	Intraop.
Elias et al.[67]	64	46	9	9	5-FU or MMC	Intraop./ Early postop.
Culliford et al.[49]	64	47	0	17	FUDV/LV	Early postop.
Witkamp et al.[64]	29	22	4	3	MMC	Intraop.
Pilati et al.[55]	34	34	0	0	MMC + cisplatin	Intraop.
Verwaal et al.[36]	105	75	12	18	MMC	Intraop.
Elias et al.[50]	35	27	3	5	MMC + 5-FU	Early postop.
Carmignani et al.[48]	27	Not spec.	Not spec.	Not spec.	MMC + 5-FU	Intraop./ Early postop.
Mahteme et al.[54]	18	16	2	0	5-FU	Early postop.
Elias et al.[51]	24	Not spec.	Not spec.	0	Oxaliplatin	Intraop.
Shen et al.[57]	77	74	3	0	MMC	Intraop.
Glehen et al.[52]	53	48	5	0	MMC	Intraop.
Glehen et al.[66]	506	466	40	0	Various	Various
Kecmanovic et al.[65]	18	14	4	0	MMC + 5-FU	Intraop./ Early postop.

Table 3. Continued

Reference	Hyper-thermia applied?	mortality	Median survival	1-year survival	2-year survival	3-year survival	5-year survival
Sugarbaker et al.[58]	No	1.7%	18 m	55%	40%	35%	_
Sugarbaker et al.[59]	No	not reported	12 m	_	_	_	_
Schneebaum et al.[56]	Yes	0%	_	_	_	_	_
Elias et al.[67]	Yes	9.3%	26 m	85%	60.1%	47.1%	27.4%
Culliford et al.[49]	No	0%	34 m	85%	60%	35%	28%
Witkamp et al.[64]	Yes	3%	20 m	82%	45%	23%	_
Pilati et al.[55]	Yes	0%	18 m	_	31%	_	_
Verwaal et al.[36]	Yes	8%	22.4 m	65%	40%	_	_
Elias et al.[50]	No	19%	_	80%	60%	30%	20%
Carmignani et al.[48]	Yes	0%	_	58%	25%	18%	_
Mahteme et al.[54]	No	0%	32 m	70%	60%	30%	30%
Elias et al.[51]	Yes	8%	_	83%	75%	65%	_
Shen et al.[57]	Yes	12%	16 m	_	_	25%	17%
Glehen et al.[52]	Yes	4%	13 m	55%	32%	_	11%
Glehen et al.[66]	Yes	4%	19.2 m	72%	_	39%	19%
Kecmanovic et al.[65]	Yes	0%	15 m	_	_	_	_

Not spec. not specified; MMC mitomycin-C; 5-FU 5-fluorouracil; Early postop. early postoperative; Intraop. intraoperative

Glehen et al.[70] analyzed the morbidity and mortality rates following 216 consecutive cytoreductive surgeries and (hyperthermic) IPEC in 207 patients with PC. Most patients suffered from ovarian, colorectal or gastric cancer. Grade III/IV toxicity was encountered in 51 patients (23.6%), digestive fistulization (6.5%) and hematological toxicity (4.5%) being the most frequent complications. Seven patients (3.4%) died from treatment-related complications.

Prognostic indicators of survival

Univariate and multivariate analyses of most series of patients with PC of colorectal origin revealed several clinical (preoperative), surgical (intra- and postoperative), and pathological factors predictive of survival. Clinical characteristics that have been univariately correlated to an improved survival are female gender,[66] younger age,[66] and clinical performance status.[57]

The most important surgical factors that have been identified as predictors of survival are: the extent of carcinomatosis encountered at laparotomy[36,48,49,51,52,58-60,65-67] and the completeness of resection.[36,48,49,51,52,54,57-60,64-66] Various scoring system were used to assess the extent of carcinomatosis. Most authors used the semi-quantitative peritoneal cancer index (PCI), described by Jacquet and Sugarbaker.[72] The PCI relies on the distribution and size of the cancer lesions throughout the abdomen. More extensive carcinomatosis or higher PCI was invariably associated with decreased survival. After the surgical cytoreduction, the size of residual disease was usually expressed as R1, meaning no macroscopic residual disease, R2a, meaning macroscopic residual disease less than 2.5 or 5 mm, or R2b, meaning macroscopic residual disease more than 2.5 or 5 mm in diameter. When reported, median survival following complete R1 resection of all macroscopic disease varied from 17.8 months to 39.0 months,[52,54,57,60,65,66] whereas the reported five-year survival rates varied from 20% till 54%.[49,52,57,66] Median survival after incomplete R2a resection resulted in median survival times of 12.5 months till 24 months,[36,52,54,57,60,66] with 5-year survival rates of between 10% and 29%.[52,59,66] When macroscopic disease of more than 5 mm in diameter had to be left behind, the reported median survival varied between 5 months and 12 months.[36,52,57,59,60] None of these patients survived for five years.[59,66] Finally, bowel obstruction[57], the presence of ascites,[57] and the presence and resection of metastatic disease to the liver were reported to be significantly correlated with imparied survival.[57,66]

Several authors have made an attempt to correlate the site of the primary tumor (colon vs. appendix vs. rectum) with prognosis.[36,48,49,58,60,66] Verwaal et al. analyzed a series of 102 consecutive patients with PC of colorectal or appendiceal origin who had been treated with cytoreductive surgery followed by hyperthermic IPEC. The 5 patients with PC of rectal origin had a median survival of 16.0 months, whereas those 82 patients with PC of colonic origin had a median survival of 21.6 months (relative risk 3.14, 95% CI 1.11-8.91, P=0.069). Similar results were reported by Cul-

liford et al., although in their patient series six out of seventeen patients with appendiceal carcinomatosis had pseudomyxoma peritonei.[49] Other authors, however, did not confirm worse survival of patients with PC of rectal origin as compared to patients with PC of colonic origin.[36,66] Sugarbaker et al. reported on the efficacy of cytoreductive surgery and IPEC in a relatively large patient series of 51 patients with colorectal carcinomatosis and 130 patients with PC of appendiceal origin. Three-year survival of the patients with PC of appendiceal origin was significantly better than that of the patients with PC of colorectal origin (73% vs. 36%, P=0.0001). Other authors, however, did not find a significant survival benefit for patients with appendiceal carcinomatosis as compared to patients with PC of colonic or rectal origin.[36,60,66] Finally, other pathological factors that have been correlated with impaired survival include poor tumor differentiation,[52,55,58-60,66] signet cell histology,[60] and lymph node involvement.[58,66]

The results of multivariate analyses on the above-mentioned clinicopathological factors were reported in five publications.[49,52,57,60,66] In four of these, the extent of disease and/or the completeness of resection were the factors most prominently related to treatment success and survival.[49,52,60,66] Shen et al. found the presence of ascites or bowel obstruction to have an even greater impact on survival.[57] In the large multi-institutional patient series of 506 patients with PC of colorectal origin, Glehen et al. furthermore identified treatment by a second procedure, age less than 65 years, and use of adjuvant chemotherapy as positive independent prognostic indicators, whereas the use of neoadjuvant chemotherapy, lymph node involvement, presence of liver metastasis, and poor histologic differentiation were negative independent prognostic indicators.[66]

Quality of life after cytoreductive surgery and (hyperthermic) intraperitoneal chemotherapy

In recent years there is an increased interest in the impact of a disease as well as its treatment on the quality of life in patients with cancer.[73] In ovarian cancer, cytoreductive surgery and adjuvant (intravenous) chemotherapy has clearly been shown to result in better survival and improved quality of life.[74,75] Only two, interrelated studies have been published focusing on quality of life after cytoreductive surgery followed by (hyperthermic) IPEC for the treatment of PC in nongynecologic malignancy. McQuellon et al.[76] investigated the quality of life of 64 patients with various peritoneal surface malignancies, 16 of whom of colonic origin, in the first year after cytoreductive surgery and (hyperthermic) IPEC. Quality of life was assessed by means of the Functional Assessment of Cancer Therapy-Colon (FACT-C) scale, analysis of various activities of daily living, the Brief Pain Inventory, the Center for Epidemiologic Studies-Depression (CES-D) scale, and the Eastern Cooperative Oncology Group (ECOG) performance status rating scale. Before surgery, patients with ascites had a significantly lower quality of life as compared to patients with-

out ascites. However, patients with ascites reported an improved overall quality of life immediately after surgery whereas those patients without ascites reported a decreased quality of life during the first three months after the surgery. From three months postoperatively onwards, quality of life improved relative to baseline. At one year after surgery, 58% of patients reported a normal performance status rating whereas 14% had to spend extra time in bed during the day due to disease- or treatment-related symptoms. The mean scores at activities of daily living, however, were still lower than the general population norm, even after successful treatment and symptom reduction.
In a second publication, McQuellon et al.[77] reported the quality of life of seventeen patients who had survived more than 3 years after cytoreductive surgery and (hyperthermic) IPEC for PC. Sixteen patients reported no limitations on moderate activities whereas ten patients described their health as very good or excellent.

Controlled studies

To date, three controlled studies have been published verifying the efficacy of cytoreductive surgery and (hyperthermic) IPEC. Of these studies, two were randomized. Mahteme et al.[54] compared eighteen patients with PC of colorectal origin, who had been treated with cytoreductive surgery and early postoperative IPEC (5-FU, Cisplatinum, or Irinotecan) as well as intravenous 5-FU-based chemotherapy, with eighteen matched control patients with PC, who received intravenous chemotherapy only. The median survival as well as the two-year and five-year survival rates of the patients who were treated with cytoreductive surgery and IPEC were significantly better than those of the control group (32 months vs 14 months, and 60% vs 10% and 28% vs 5%, respectively (P=0.01)). The authors concluded, that, although selection bias may have influenced the results, the results indicate that cytoreductive surgery followed by IPEC can be beneficial and result in complete remission of the disease for a prolonged period of time.
The seemingly better results of the aggressive surgical approach with respect to survival led Verwaal et al.[36] to conduct a randomized controlled trial (RCT), investigating the efficacy of this treatment as compared to merely palliative treatment, consisting of systemic chemotherapy and surgery when indicated. One hundred-and-five patients with established PC of colorectal or appendiceal origin, without hematogenous metastases, were randomized to be treated with either cytoreductive surgery and hyperthermic IPEC using MMC followed by systemic 5-FU/LV based chemotherapy or systemic chemotherapy alone and palliative surgery when necessary. Despite the rather high postoperative mortality of 8%, median survival after surgical cytoreduction and (hyperthermic) IPEC was 22.3 months, which was significantly better (P=0.032) than the median survival of 12.6 months, obtained in the control arm. Several comments on this RCT seem justified. Firstly, in both treatment arms patients had surgical interventions, the effect of which remains un-

known. Secondly, patients in the standard treatment arm were treated with 5-FU-based chemotherapy. Several new cytostatic agents, such as irinotecan and oxaliplatin have been introduced. When combined with 5-FU/LV, both irinotecan and oxaliplatin have been shown to be superior to 5-FU/LV alone in advanced CRC.[78-81] Therefore, it cannot be excluded that the observed survival difference between the patients treated with cytoreductive surgery, (hyperthermic) IPEC and systemic 5-FU/LV versus the patients treated with systemic 5-FU/LV and palliative surgery, may become less pronounced, when chemotherapy is changed to a combination of 5-FU/LV and irinotecan and/or oxaliplatin.
The second RCT was conducted by Elias et al.,[50] who randomized patients with established PC to be treated with cytoreductive surgery with or without early postoperative IPEC. Unfortunately, due to difficulties in patient recruitment, the trial was prematurely terminated after 35 patients. Two-year survival rate after cytoreductive surgery was 60%. Early postoperative IPEC had no measurable effect on treatment outcome.

Conclusions

Improved insights into the mechanisms and incidence of intraperitoneal spread of CRC have contributed to a better understanding and a different perception of the pathologic basis of PC. Even though the reported incidence rates of intraperitoneally exfoliated cancer cells during resection of primary tumors varied widely, the presence of free tumor cells in the peritoneal cavity of some patients was repeatedly demonstrated in all studies. Although the presence of exfoliated tumor cells in the peritoneal cavity, similar to micrometastases in blood or bone marrow,[82-84] may not be an independent prognostic factor, it seems plausible that these tumor cells may indeed contribute to intraperitoneal treatment failure. In fact, in two studies a correlation was found between the presence of free tumor cells in the peritoneal cavity and intraperitoneal tumor recurrence.[12,18]
Despite the favorable results of cytoreductive surgery and adjuvant (hyperthermic) IPEC in patients with PC of colorectal origin, the results should be interpreted with caution for several reasons. Firstly, the reported trials differed significantly with regard to patient selection. In some trials patients with hematogenous metastases were eligible for inclusion, whereas other patient series also included patients with appendiceal carcinomatosis or pseudomyxoma peritonei. The latter two disease entities are may have a relatively favorable prognosis as compared to PC of colorectal origin. Secondly, the design of the combination treatment inflicted on the patients differed widely with regard to the timing of the IPEC (preoperative, intraoperative, postoperative or combinations), the method of administration of the chemotherapy (open or closed abdomen), the cytostatic agent(s) utilized and whether or not

chemotherapy was given under hyperthermic conditions. Thirdly, the patient series published to date all come from specialized tertiary referral centers.
Nonetheless, the results in terms of survival after cytoreductive surgery and (hyperthermic) IPEC appear much better than those obtained in historical controls and have, indeed, been shown to be superior to conventional chemotherapy-based treatment in one RCT.[36] Given the high morbidity and mortality rates of cytoreductive surgery and (hyperthermic) IPEC as well as the high failure rate of 80%, it is of the utmost importance to select only those patients for this treatment modality that could benefit the most. Univariate and multivariate analysis of several clinicopathological parameters repeatedly showed the extent of the carcinomatosis and, consequently, the extent of surgery to be the most important factors related to postoperative morbidity and mortality. In all patient series, postoperative morbidity and mortality was predominantly determined by surgery-related factors, such as the number of anastomoses, peritonectomy procedures, and the amount of blood loss. Toxicity clearly attributable to the hyperthermic IPEC was relatively rare. Furthermore, despite the differences in timing and methods of intraperitoneally administering chemotherapy, the completeness of resection has proven to be the most important prognostic factor predictive of survival in almost all patient series reported to date. Patients, in whom complete resection of all macroscopic disease was not feasible, invariably showed a dismal prognosis similar to that of historical controls.[61] Interestingly, complete resection frequently resulted in survival rates comparable to those obtained after resection of liver metastases.[85] Cytoreductive surgery and (hyperthermic) IPEC should therefore only be offered to patients with limited and resectable disease.
According to Sugarbaker as well as Verwaal et al. the time has come to accept cytoreductive surgery and (hyperthermic) IPEC as one of the standard treatments for patients with limited carcinomatosis.[86,87] Sugarbaker argues that the acquisition of level I evidence by meta-analysis of data from several well-designed RCTs may not be necessary for experimental therapies to mature into standard of care. For example, while there has never been an RCT confirming the superiority of resection of liver metastases of colorectal origin over systemic chemotherapy, surgery is generally accepted as standard of care in selected patients with liver metastases of colorectal origin. Others recognize the efficacy of the cytoreductive surgery but argue that, based on only one RCT, it is too early to accept it as part of a procedure in which (hyperthermic) IPEC is applied.[88] With the completeness of resection being the most important prognostic factor, it could, indeed, be questioned whether (hyperthermic) IPEC is mandatory after complete resection. For that reason Mansfield[88] recognizes the need to move forward and pleads for a new multicenter RCT, comparing cytoreductive surgery followed by (hyperthermic) IPEC and systemic chemotherapy with cytoreductive surgery and systemic chemotherapy alone, resembling the design of the RCT conducted by Elias et al.[50] In fact, such a trial is currently considered at the Netherlands Cancer Institute.

Given the small number of medical centers worldwide currently practicing cytoreductive surgery and (hyperthermic) IPEC for the treatment of PC, larger-scale application of this treatment modality may be challenging. Both the surgery and the administration of (hyperthermic) IPEC are technically demanding procedures, for which a learning curve exists.[86] The further implementation of cytoreductive surgery and (hyperthermic) IPEC for the management of PC of colorectal origin should therefore be pursued with caution and should still be applied as part of controlled trials.

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3

Radioimmunotherapy of experimental peritoneal carcinomatosis of colorectal origin

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Besides the lymphatic and hematogenous routes of dissemination, colorectal cancer frequently gives rise to intraperitoneal (i.p.) spread of tumor cells. Microscopic i.p. dissemination of cancer cells can be demonstrated in 10% to 47% of patients undergoing surgery for colorectal cancer, depending on the detection method used.[1,2] This so-called seeding of tumor cells may lead to peritoneal carcinomatosis (PC), a condition characterized by the deposition of multiple metastatic nodules on the peritoneal surfaces. Although the clinical picture of patients with metastatic colorectal cancer is frequently dominated by liver metastases, peritoneal metastases can be a major source of morbidity[3] and prognosis of patients with this condition is dismal, with a reported median survival of 6 months.[4] Although PC is generally considered a terminal condition only to be palliated, there is a renewed interest in the subgroup of patients with metastatic disease limited to the peritoneal cavity. In the past two decades, a new treatment modality was developed, combining surgical cytoreduction and i.p. chemotherapy with the aim of achieving locoregional, i.e., i.p. tumor control. Although encouraging results in terms of survival have been reported, the morbidity and mortality rates of this aggressive approach are considerable and in many cases treatment is neither radical nor curative. This warrants the development of new, more specific treatment modalities for PC, which could ideally also be used in an adjuvant setting.

Radioimmunotherapy using radiolabeled monoclonal antibodies (MAbs) directed against tumor-associated antigens offers the opportunity to selectively irradiate tumor cells while normal tissues are relatively spared. Promising results have been reported in patients with hematological malignancies, presumably due to their high intrinsic radiosensitivity and the relatively good access of the radiolabeled antibodies to the cancer cells.[5,6] Solid cancers, however, proved to be much less sensitive to this form of treatment due to various reasons, including a limited vascular supply, heterogeneous uptake of the antibody in the tumor, and elevated interstitial pressure in combination with a relatively long transport distance in the interstitium.[7] These barriers especially apply to larger tumors. Therefore, minimal residual disease (and occult metastases) is generally considered the optimal setting for therapy using radiolabeled MAbs.8 In this regard, promising results have been reported by Epenetos et al.,[9] who observed a ten-year survival rate of over 78% after treatment with ^{90}Y-labeled HMFG1 MAb in 21 patients with ovarian cancer, who had achieved complete remission after cytoreductive surgery and platinum-based chemotherapy.

Although colorectal carcinoma is not very radiosensitive,[10] the potential of radioimmunotherapy has been subject of investigation in several preclinical[11-14] and clinical studies[15-19]. The majority of preclinical studies investigated its efficacy in nude mice bearing subcutaneous xenografts. These studies showed that radioimmunotherapy may be superior to equitoxic chemotherapy.[11] Therefore, it seemed worthwhile to ascertain the efficacy of radioimmunotherapy in early-stage peritoneal carcinomatosis of colorectal origin.[20-22]

Here we report the results of studies investigating the application of ^{131}I-based radioimmunotherapy using the anti-CEA MAb MN-14 in an experimental model of small peritoneal metastases of colonic origin.

Materials and methods

Animals

Male nude BALB/c mice, 6-8 weeks old, weighing 20-25 grams were used in the experiments. Mice were accustomed to laboratory conditions for at least one week before experimental use and were housed under clean, nonsterile standard conditions in filter-topped cages (5 mice per cage), with free access to animal chow (Snif Voer, Soest, The Netherlands) and water. All experiments were approved by and carried out in accordance with the guidelines of the local Animal Welfare Committee.

Cell line

The human colon carcinoma cell line LS174T was obtained from the American Type Culture Collection (Rockville, MD). LS174T is a rapidly growing, moderately to well differentiated human colon carcinoma cell line, characterized by a moderate level of CEA-expression (114 ng/10^7 cells; 5,000 – 10,000 epitopes/cell).[11,23] LS174T was cultured and maintained as monolayers on plastic in RPMI-1640 medium (GIBCO, BRL Life Sciences Technologies, The Netherlands) supplemented with 10% fetal calf serum (GIBCO), 2 mM L-glutamine, penicillin (100 units/mL) and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere with 5% CO_2. Prior to inoculation, tumor cells were washed with 0.9% sodium chloride, disaggregated with 0.25% trypsin and resuspended in complete medium to the appropriate concentration.

Monoclonal antibody

The murine MN-14 MAb is a high-affinity (K_a = 10^9 liters/mol) class-III anti-CEA IgG_1 antibody, produced by a hybridoma cell line culture, kindly provided by Immunomedics, Inc. (Morris Plains, New Jersey, USA).[24] The antibodies were purified by protein A chromatography, as described previously.[25] Purity was checked by fast protein liquid chromatography (FPLC) on a Phenomenex Biosep 3000 column, using phosphate buffered saline (PBS, pH 7.2) as eluens (1 mL/min).

Radioiodination

Antibodies were radioiodinated using the iodogen-method.[26] Briefly, antibodies (1.0 – 1.5 mg) and ^{125}I or ^{131}I were incubated at room temperature in 85 µl of PBS (0.10 M, pH 7.4) in a glass vial, coated with 50-100 µg iodogen. After ten to twelve minutes, the reaction was stopped by adding 100 µl of a saturated tyrosine solution. The reaction mixture was subsequently eluted on a PD-10 column, eluted with PBS, 0.5% BSA. Labeling efficiency of all radioiodination reactions varied between 74% and 92%. Radiochemical purity (RCP) was determined using instant thin-layer chromatography (ITLC) on silicagel strips (Gelman Sciences, Ann Arbor, MI) using 0.10 M citrate buffer (pH 6.0) as the mobile phase. RCP's of all preparations used in the experiments always exceeded 98%. Immunoreactivity of the radioiodinated MN-14 preparations was essentially determined as described by Lindmo et al.[27] Briefly, a fixed amount of labeled antibody (10,000 cpm) was incubated (6 hours, 37 °C) with increasing concentrations of LS174T tumor cells (1.2×10^6 – 20×10^6 cells/mL) in 0.5 mL binding buffer (RPMI medium containing 0.5% BSA and 0.05% NaN3). A duplicate of the lowest cell concentration was incubated in the presence of an excess unlabeled antibody to correct for non-specific binding. After six hours of incubation at 37°C, the cells were spun down (500 g, 5 min) and activity in the pellet was determined in a well-type gamma-counter. The inverse of the tumor cell bound fraction was plotted against the inverse of the cell concentration and the immunoreactive fraction (IRF) was calculated from the y-axis abscis. Immunoreactivity of the radioiodinated MN-14 preparations used in the experiments varied between 70% and 90%. Labeled antibody preparations were administered within two hours after the labeling procedure.

Experimental model of peritoneal carcinomatosis

The metastatic pattern of LS174T tumor cells after i.p. inoculation was studied, as described by Lopes Cardozo et al.[28] Mice were inoculated intraperitoneally with 1.0×10^6 LS174T cells, suspended in 500 µl of RPMI-1640 medium in a 2.5 mL syringe using a 23 gauge needle. Mice were anaesthetized, bled and cervically dislocated at weekly intervals. The abdominal cavity was conscientiously inspected. Spleen, liver and lungs were removed for routine histopathological H&E-staining and immunohistochemical staining using a rabbit-anti-human anti-CEA polyclonal antibody (A 0115, DakoCytomation, Glostrup, Denmark).[29]

Effect of the antibody protein dose on biodistribution

To determine the effect of antibody-protein dose on the biodistribution of radioiodinated MN-14, mice received 10 µCi ^{131}I-labeled MN-14 intraperitoneally at eight different MN-14 protein doses ten days after i.p. inoculation of 1.0×10^6 LS174T tumor cells (five mice per group). Specific activity of the radioiodinated MN-14 preparation

was 4.3 μCi/μg. Protein doses were adjusted by adding unlabeled MN-14 to a fraction of the same ^{131}I-labeled MN-14 preparation and amounted 1, 10, 15, 20, 25, 30, 50 or 100 μg (five mice per group). Mice were dissected 72 hours after administration of the radiolabeled antibody. Tumor, blood, liver, spleen, kidney, intestine, lung and muscle tissues were sampled, gently blotted dry, and weighed. Activity was measured in a shielded well-type gamma-counter (Wizard, Pharmacia-LKB, Sweden). To correct for physical decay and to calculate uptake of the radioiodinated antibody in each sample as a fraction of the injected dose, aliquots of the injected dose were counted simultaneously. The results were expressed as percentage of the injected dose per gram tissue (% ID/g).

Effect of the route of administration on biodistribution

To assess the effect of the route of administration on the biodistribution of radioiodinated MN-14, mice were inoculated intraperitoneally with 1.0 x 10^6 LS174T tumor cells suspended in RPMI-1640 (500 μl). Ten days later, the mice received 10 μCi ^{131}I-labeled murine MN-14 intraperitoneally and 5 μCi ^{125}I-labeled MN-14 intravenously. Specific activities of the radioiodinated MN-14 preparations were 2.1 μCi/μg and 1.1 μCi/μg, respectively. Total protein dose of each preparation was adjusted to 10 μg per mouse (total: 20 μg per mouse). Mice were euthanized by O_2/CO_2-asphyxia and dissected at one, two, four, eight, 24, 48, 72, 96 and 192 hours after the administration of the radiolabeled antibody preparations (five mice per group). The same tissues as described above were sampled, weighed and counted in a well-type gamma-counter. Activity was expressed as % ID/g. Biodistribution data were processed using the Medical Internal Radiation Dose (MIRD) scheme[30], in order to estimate the radiation dose that a therapeutic activity dose of ^{131}I-labeled MN-14 would guide to the i.p. tumor deposits.

Radioimmunotherapy studies

Ten days after i.p. tumor inoculation, mice received i.p. injections of ^{131}I-labeled MN-14 at escalating activity doses: 62.5, 125, 250 or 500 μCi (ten mice per group). Activity dose escalation was terminated at 500 μCi, since this was previously determined as the maximal tolerated activity dose of ^{131}I-labeled IgG antibody after i.p. administration (i.e. the dose level below the lowest activity dose level that caused death or loss of more than 20% of initial total body weight.)[31] Control mice received unlabeled MN-14 or 500 μCi ^{131}I-labeled nonspecific IgG-control antibody.[32] Preparations were administered in 500 μl PBS, 0.5% BSA. Specific activity of the radioiodinated MN-14 and control antibody preparations was 38 μCi/μg and 27 μCi/μg respectively. Protein dose was adjusted to 20 μg by adding unlabeled MN-14 or unlabeled control antibody to a fraction of the primary radioconjugate preparations. Mice were monitored daily and body weight and abdominal circumference was measured twice per week (MJK). Mice were euthanized by cervical dislocation when the abdominal circumference had increased

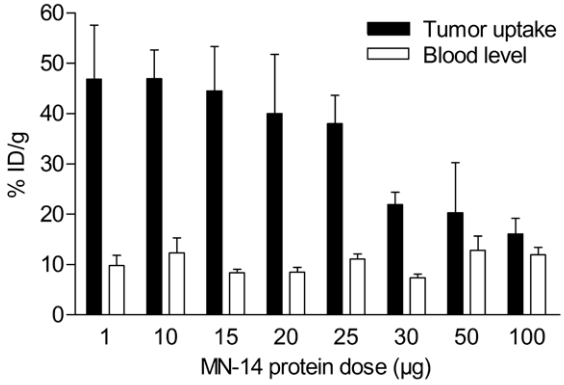

Figure 1a
Blood levels and uptake in tumor after administration of escalating protein doses of MN-14. Tissues were sampled 72 hours post-injection of the radioiodinated MN-14 antibody. Values are given as means ± standard deviation (five mice per group).

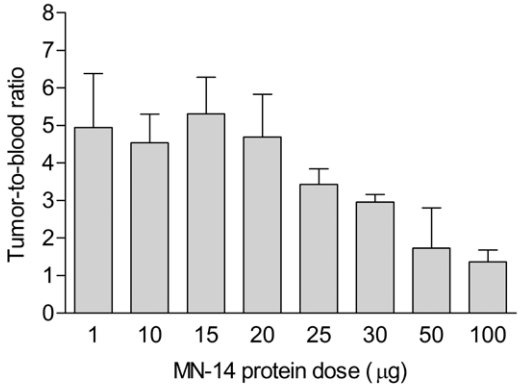

Figure 1b
Tumor-to-blood ratios after administration of escalating protein doses of MN-14. Tissues were sampled 72 hours post-injection of the radioiodinated MN-14 antibody. Values are given as means ± standard deviation (five mice per group).

by 10% as compared to the abdominal circumference measured on the day of tumor inoculation. All i.p. tumor deposits were meticulously dissected and weighed.

Results

Experimental model of peritoneal carcinomatosis

The first macroscopic LS174T tumor deposits in the peritoneal cavity were observed seven days after i.p. inoculation, as was described by Esteban et al.[13] Tumor deposits were small (1-3 mm in diameter) and predominantly located in the upper abdomen in the liver hilum, the greater omentum and adjacent to the spleen. Bulky disease, including macroscopic metastases on the diaphragm and hemorrhagic ascites was ob-

served three to five weeks after tumor cell inoculation. Routine histopathological as well as immunohistochemical examination of the liver, spleen and lungs revealed no microscopic metastases at any time. Histopathological examination of the diaphragm showed deep invasion by tumor, without reaching the pleural surface.

Protein-dose escalation

To determine the maximum antibody protein dose that can be used for radioimmunotherapy, the biodistribution of escalating protein doses of intraperitoneally administered ^{131}I-MN-14 was assessed 72 hours p.i. The uptake in the tumor deposits, blood levels as well as tumor-to-blood ratios as function of the MN-14 protein dose are presented in Figure 1a-b. The biodistribution of the radioiodinated MN-14 antibody was not affected by antibody protein doses up to 25 μg per mouse, whereas tumor-to-blood ratios were similar up to doses of 20 μg. At higher doses, a lower tumor-to-blood level and a reduced uptake in the i.p. tumor deposits was observed, presumably due to saturation of the accessible CEA-epitopes in the tumor deposits. Uptake in other tissues was similar at all protein doses (data not shown).

Biodistribution of radioiodinated MN-14

The biodistribution of radioiodinated MN-14 in this model was studied following i.v. or i.p. administration and the results are summarized in Table 1a-b. The blood levels of radioiodinated MN-14 are presented in Figure 2a. At 8 hours p.i the blood level was still higher after i.v. administration. From 24 hours onwards the blood level of the intraperitoneally injected ^{131}I-MN-14 and the intravenously injected ^{125}I-MN-14 were similar. In all other normal tissues higher uptake was observed after i.v. administration until 24 hours p.i. (Table 1a and 1b). After both i.v. and i.p. administration the uptake of the radiolabeled antibody in tumor deposits was relatively high. In the first 24 hours following administration, the i.p. route led to higher uptake in the tumor than the i.v. route (Figure 2b; Table 1a and 1b). From 48 hours p.i. onwards tumor uptake was in the same range for both routes of administration. From two hours onwards after i.p. administration, uptake in tumor was approximately 50% ID/g. Highest uptake in the tumor after i.p. administration (58.5 ± 6.8 % ID/g, 24 hours p.i.) was similar to the maximum uptake after i.v. administration (47.9 ± 11.5 % ID/g, 48 hours p.i., two-tailed P = 0.19, Mann-Whitney test).
In Figure 2c the tumor-to-blood ratios following both routes of administration are depicted, indicating that in the first 24 hours the tumor-to-blood ratio after i.p. administration was higher than that after i.v. administration (two-tailed P = 0.03, Mann-Whitney test comparing tumor-to-blood ratios at 24 hours p.i.).
When corrected for physical decay of ^{131}I, the areas under the curve (AUCs) for blood were similar for both routes of administration (15.8 versus 15.0 h x μCi/g), whereas the AUC for tumor after i.p. administration was somewhat higher (66.6

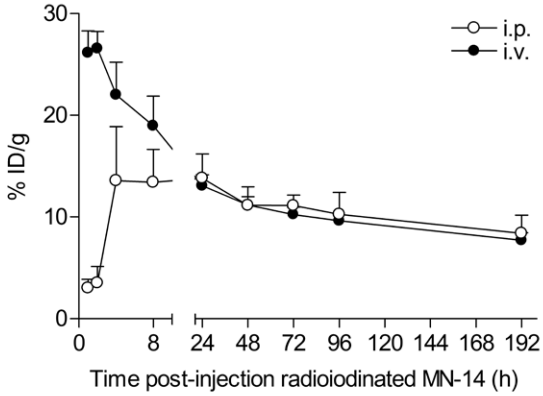

Figure 2a
Blood levels of radioiodinated MN-14 after i.v. versus i.p. administration in nude mice bearing peritoneal LS174T tumor xenografts. Values are given as means ± standard deviation (five mice per group).

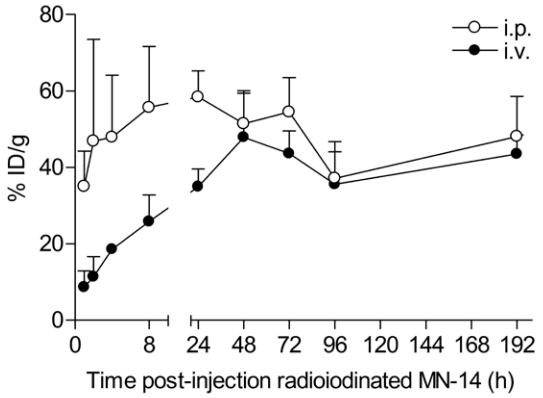

Figure 2b
Uptake of radioiodinated MN-14 in peritoneal LS174T tumor xenografts after i.v. versus i.p. administration. Values are given as means ± standard deviation (five mice per group).

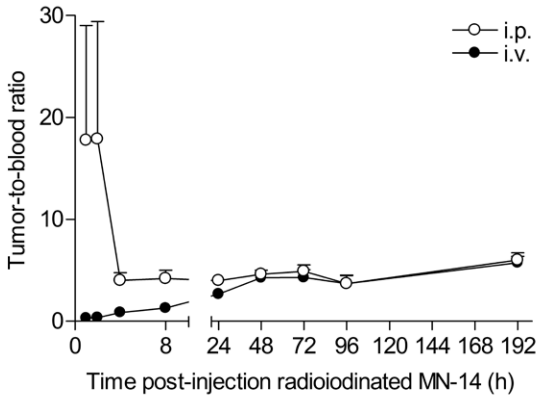

Figure 2c
Tumor-to-blood ratios after i.v. versus i.p. administration of radioiodinated MN-14 in mice bearing peritoneal LS174T tumor xenografts. Values are given as means ± standard deviation (five mice per group).

Table I-a. Tissue distribution of intraperitoneally administered ^{131}I-MN-14 in nude mice bearing intraperitoneal LS174T colon cancer xenografts.

Organ	Time post-injection radioiodinated MN-14 (hours)								
	1	2	4	8	24	48	72	96	192
Tumor	35.1 ± 9.3	46.9 ± 26.7	47.9 ± 16.3	55.7 ± 15.9	58.5 ± 6.8	51.5 ± 8.7	54.5 ± 9.0	37.2 ± 9.6	48.1 ± 10.5
Blood	3.0 ± 0.9	3.5 ± 1.6	13.6 ± 5.4	13.4 ± 3.2	13.8 ± 2.4	11.1 ± 1.8	11.1 ± 1.0	10.3 ± 2.2	8.4 ± 1.8
Muscle	3.3 ± 1.2	7.0 ± 2.9	5.4 ± 1.8	3.1 ± 0.9	1.8 ± 0.2	1.3 ± 0.1	1.1 ± 0.1	1.0 ± 0.2	0.8 ± 0.1
Lung	2.4 ± 0.2	2.2 ± 0.9	10.2 ± 3.9	10.3 ± 3.2	8.0 ± 1.8	6.8 ± 0.7	6.4 ± 0.4	5.8 ± 1.5	4.8 ± 1.3
Spleen	4.4 ± 0.4	2.0 ± 1.1	5.1 ± 3.0	4.1 ± 1.3	3.7 ± 0.6	2.7 ± 0.7	2.8 ± 0.5	2.0 ± 0.4	1.7 ± 0.3
Kidney	1.6 ± 0.4	1.5 ± 0.6	4.4 ± 1.7	4.3 ± 1.1	4.1 ± 0.6	3.2 ± 0.5	3.1 ± 0.4	2.8 ± 0.6	2.1 ± 0.4
Liver	2.6 ± 0.5	2.0 ± 0.5	4.7 ± 2.0	4.2 ± 1.0	4.7 ± 0.6	3.6 ± 0.6	3.9 ± 0.5	3.9 ± 1.0	2.6 ± 0.6
Intestine	3.2 ± 1.2	3.4 ± 1.7	4.6 ± 3.3	2.0 ± 0.5	2.1 ± 0.6	1.7 ± 0.3	1.5 ± 0.2	1.6 ± 0.6	1.0 ± 0.2

Values are given as means ± standard deviation (five mice per group)

Table I-b. Tissue distribution of intravenously administered ^{125}I-MN-14 in nude mice bearing intraperitoneal LS174T colon cancer xenografts.

Organ	Time post-injection radioiodinated MN-14 (hours)								
	1	2	4	8	24	48	72	96	192
Tumor	8.6 ± 4.3	11.4 ± 5.3	18.6 ± 0.8	25.9 ± 6.9	34.9 ± 4.7	47.9 ± 11.5	43.6 ± 5.9	35.6 ± 8.6	43.5 ± 5.0
Blood	26.1 ± 2.2	26.6 ± 1.7	22.0 ± 3.2	19.0 ± 2.9	13.1 ± 1.1	11.2 ± 0.8	10.2 ± 0.7	9.6 ± 0.5	7.7 ± 0.7
Muscle	0.9 ± 0.3	0.9 ± 0.2	0.9 ± 0.2	1.1 ± 0.1	1.3 ± 0.2	1.2 ± 0.1	1.1 ± 0.1	1.0 ± 0.0	0.7 ± 0.0
Lung	13.4 ± 3.5	14.7 ± 1.6	18.2 ± 3.1	14.7 ± 1.6	7.9 ± 1.5	6.8 ± 0.8	6.0 ± 0.8	5.4 ± 0.7	4.3 ± 0.8
Spleen	6.4 ± 1.7	6.3 ± 0.8	6.2 ± 1.6	5.8 ± 1.6	3.7 ± 0.5	2.7 ± 0.4	2.6 ± 0.5	2.0 ± 0.2	1.6 ± 0.2
Kidney	6.7 ± 0.9	6.8 ± 0.6	7.1 ± 1.0	6.1 ± 0.9	4.0 ± 0.4	3.2 ± 0.2	2.9 ± 0.3	2.7 ± 0.3	2.0 ± 0.1
Liver	10.0 ± 2.4	8.7 ± 1.5	7.8 ± 1.1	6.4 ± 1.1	4.7 ± 0.4	3.5 ± 0.3	3.6 ± 0.3	3.6 ± 0.6	2.5 ± 0.4
Intestine	3.2 ± 1.2	3.4 ± 1.7	4.6 ± 3.3	2.0 ± 0.5	2.1 ± 0.6	1.7 ± 0.3	1.5 ± 0.2	1.6 ± 0.6	1.0 ± 0.2

Values are given as means ± standard deviation (five mice per group)

versus 53.1 h x μCi/g). Dosimetric analysis using the MIRD scheme[30] indicated that i.p. radioimmunotherapy using 500 μCi of ^{131}I-MN-14 would guide 317 Gy to tumors of 10 mg or 2.6 mm in diameter.

Radioimmunotherapy

Eight mice (13%) developed subcutaneous tumors and were excluded from the analysis. There was no mortality due to treatment. Three mice with excessive tumor growth had to be euthanized before the abdominal circumference had increased by 10%. The change in body weight at the time the abdominal circumference had increased by 10% is depicted in a scatter plot in Figure 3a. These data show that about 50% of mice had gained weight, while others had lost weight, indicating that weight change cannot be used as a predictor of therapeutic efficacy in this model. Abdominal circumference, however, proved to be a useful and reproducible indicator of i.p. tumor growth. When the abdominal circumference had increased by 10%, all mice showed marked signs of i.p. tumor growth. Figure 3b shows the mean tumor weight at the time abdominal circumference had increased 10% according to group. It varied between 2.2 ± 0.6 g) in mice that received 500 μCi of ^{131}I-MN-14 and 3.9 ± 1.1 g) in mice that received 62.5 μCi of ^{131}I-MN-14 (P=0.13, one-way ANOVA-test).

Median survival of the mice that received unlabeled MN-14 was 38 days (range 28-42). Survival was significantly improved with increasing activity doses of ^{131}I-labeled MN-14, as shown in a Kaplan-Meier plot in Figure 3c. Median survival of the mice treated 62.5 μCi, 125 μCi, 250 μCi or 500 μCi of ^{131}I-MN-14 was 42 days (range 42-52; P= 0.03), 49 days (32-52; P=0.03), 63 days (59-104; P<0.0001) and 109 days (46-..., P<0.0001) respectively (Log rank test compared with unlabeled MN-14 control group). Median survival of the mice treated with 500 μCi ^{131}I-labeled control antibody was 52 days (46-73, P=0.002), which is comparable to that after treatment with 125 μCi ^{131}I-MN-14.

In the group treated with 500 μCi ^{131}I-MN-14, there were five long-term survivors (>100 days). Of these, one mouse was still alive without evidence of tumor growth five months after tumor cell inoculation. This mouse was euthanized and dissected. At dissection there was no macroscopic tumor. The macroscopic appearance of the abdominal cavity of this mouse is shown in Figure 3d in conjunction with a mouse euthanized at the time its abdominal circumference had increased by 10% due to bulky i.p. tumor growth. However, routine histopathological and immunohistochemical examination of the greater omentum, diaphragm, of random biopsies of the peritoneum and of the pancreas, liver, lungs en spleen revealed one micrometastasis in the pancreas (Figure 3e).

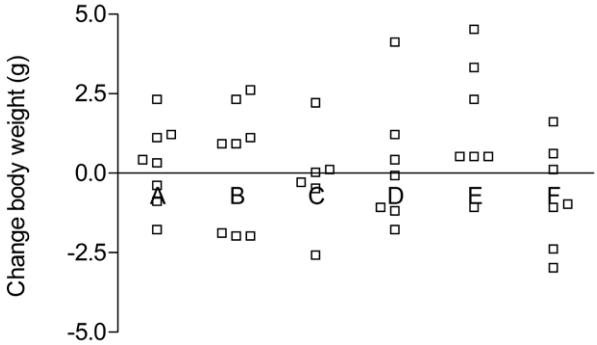

Figure 3a
Scatter plot of body weight change at the time of 10% increase of abdominal circumference in mice bearing peritoneal LS174T tumor xenografts after 500 µCi ^{131}I-MN-14 (A), 250 µCi ^{131}I-MN-14 (B), 125 µCi ^{131}I-MN-14 (C), 62.5 µCi ^{131}I-MN-14 (D), unlabeled MN-14 (E) or 500 µCi ^{131}I-labeled irrelevant antibody (F).

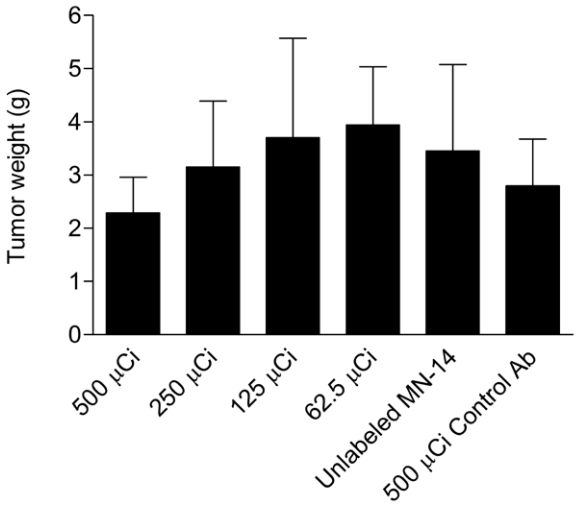

Figure 3b
Tumor weight at the time of 10% increase of abdominal circumference in mice bearing peritoneal LS174T tumor xenografts. Values are given as means ± standard deviation. Variation between the groups is not statistically significant (one-way ANOVA test; P=0.13).

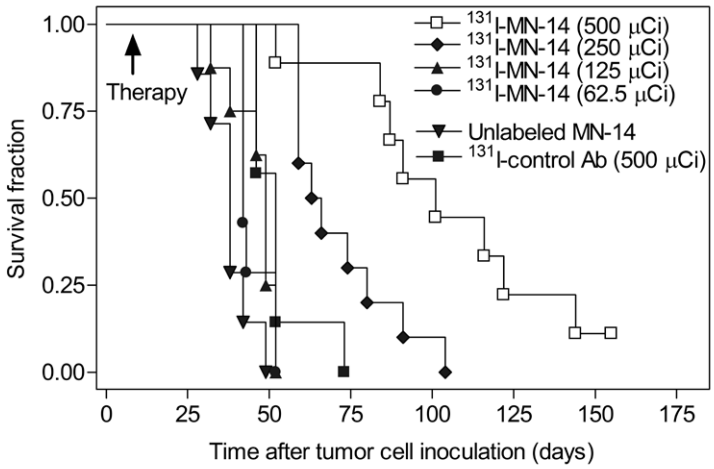

Figure 3c
Kaplan-Meyer survival plot of mice bearing peritoneal LS174T tumor xenografts after i.p. administration of escalating activity doses of ^{131}I-labeled MN-14, unlabeled MN-14 or ^{131}I-labeled irrelevant antibody (six to nine mice per group). (Log-rank test for trend $\chi^2 = 20.75$, $P < 0.0001$).

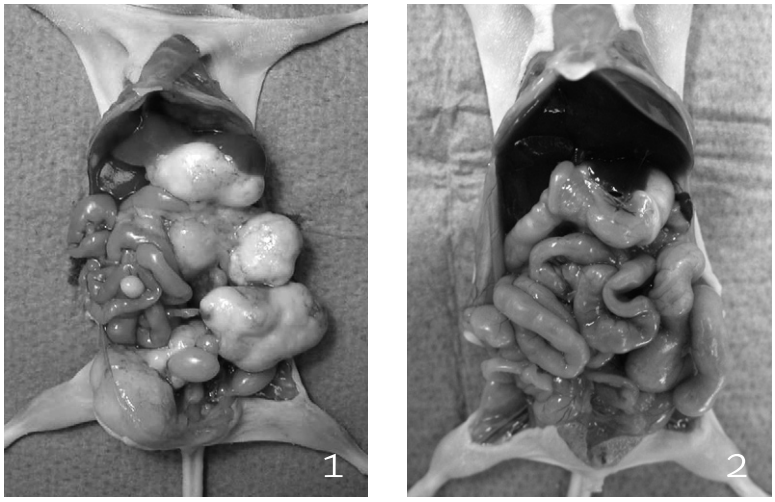

Figure 3d
The effect of radioimmunotherapy on colon cancer xenografts in the peritoneal cavity. (1) Bulky i.p. tumor growth at the time the abdominal circumference had increased by 10%. (2) Macroscopic appearance of the abdominal cavity at dissection of a mouse five months after i.p. treatment with 500 μCi ^{131}I-MN-14. No macroscopic tumor deposits were present.

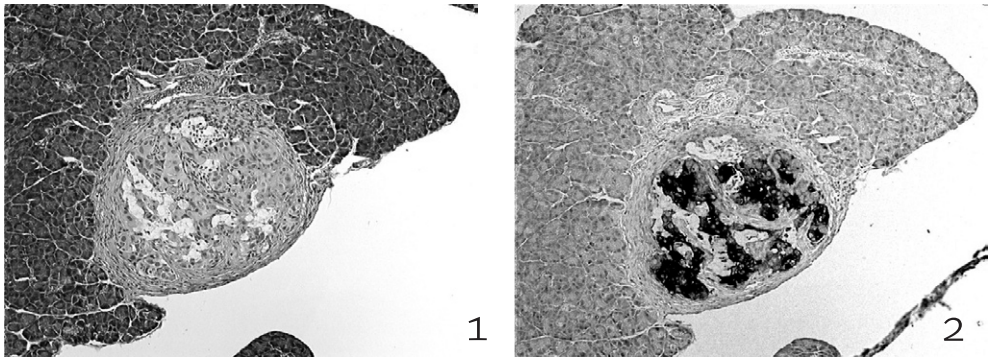

Figure 3e
H&E-staining (1) and anti-CEA-immunohistochemical (2) staining of the pancreas of the mouse shown in Figure 3d-2, revealing a micrometastasis of moderately differentiated adenocarcinoma (magnification 100x).

Discussion

The primary aim of this study was to assess the therapeutic efficacy of radioimmunotherapy of small peritoneal metastases of colorectal origin in an experimental model. The radioiodinated anti-CEA MAb MN-14 preferentially accumulated in i.p. LS174T tumor xenografts. Quantitative analysis of the biodistribution data indicated that the i.p. route of administration resulted in a 25% higher radiation dose to the tumor as compared to the i.v. route of administration. I.p. radioimmunotherapy using ^{131}I-labeled MN-14 proved to be very effective in delaying the growth of the tumor deposits, even at the lowest activity dose (62.5 μCi).

The i.p. inoculation of tumor cells is a commonly used method to induce i.p. tumor growth mimicking some of the pathological features as seen in patients with peritoneal carcinomatosis, including preferential seeding to the greater omentum and the diaphragm and the development of hemorrhagic ascites in the later stages.[33] The human colon carcinoma cell line LS174T used in the described studies is an extensively used and well-characterized cell line.[11,13,23,34-36] It is characterized by a moderately high CEA-expression and an aggressive growth pattern, which was reflected in a substantial number of subcutaneous tumors in the present studies (13%). The high-affinity class-III anti-CEA murine monoclonal IgG_1 antibody, MN-14, like other anti-CEA IgG antibodies is internalized only slowly and to a limited extent in CEA-expressing cancer cells.[37,38] It was used since it has already been successfully exploited in experimental radioimmunotherapy models.[11,39] In addition, a humanized version of this MAb has been developed and successfully used in patients with CEA-expressing cancers.[15, 12, 40-32] Its high affinity, however, is reflected in a higher propensity to form immune complexes with circulating CEA in patients with elevated CEA-levels.[41]

Before entering radioimmunotherapy studies, the present model was optimized with regard to two parameters, namely the maximum antibody protein dose still associated with optimal biodistribution, as previously described by Boerman et al.,[42] and the optimal route of administration of the radiolabeled antibody.
As demonstrated by Steffens et al.[43] in a nude mouse model of renal cell carcinoma, tumor-associated antigens can be saturated at higher antibody protein doses. The small volume of the i.p. tumor nodules in the present model limited the maximum MN-14 protein dose to 20-25 μg. At higher protein doses the uptake in the tumor nodules decreased (38 ± 5.6% ID/g at 25 μg versus 22 ± 2.4% ID/g at 30 μg) and tumor-to-blood ratio decreased as well (4.7 ± 1.1 at 20 μg versus 3.4 ± 0.4% ID/g at 25 μg), indicating that at higher antibody protein doses tumor-associated CEA was saturated. At higher protein doses the therapeutic efficacy will thus decrease, due to the reduced radiation dose to the tumor.[42]
The rationale of i.p. administration of radiolabeled antibodies in models of peritoneal carcinomatosis is twofold: firstly, due to a gradual delivery from the abdominal cavity to the circulation, the radiation dose to the blood and consequently the red marrow (i.e., the dose-limiting organ) may be reduced. Secondly, i.p. administration may lead to a higher uptake in i.p. tumor xenografts and may thus be more effective.[44] A few studies have been published, comparing the i.v. with i.p. administration of radiolabeled MAbs for the detection or treatment of experimental and clinical peritoneal carcinomatosis.[14,45-48] In the majority of these studies ovarian cancer models were used. Most studies reported the i.p. route of administration to be advantageous with regard to uptake in tumor and tumor-to-normal-tissue ratios. On the other hand, i.v. administration may result in a more homogeneous uptake in i.p. xenografts, as compared to i.p. administration.[47] This may especially apply to larger tumors, as was elegantly demonstrated by Ito et al.,[47] who studied the intratumoral distribution of the anti-CEA MAb C110 after i.p. and i.v. administration by autoradiography in nude mice bearing relatively large (2-10 mm) i.p. LS174T tumor nodules. I.p. administration resulted in a high uptake in the tumor periphery and a considerably lower uptake in the tumor center, whereas an almost homogenous distribution throughout the tumor nodules was seen after i.v. administration. In the present study, tumor nodules were much smaller (1-3 mm) and i.p. administration led to a significantly higher uptake in the tumor nodules during the first 24 hours p.i. In our view, when tumor nodules are relatively small, i.p. administration is therefore preferable for radioimmunotherapy. The blood levels were similar from 24 hours p.i. onwards and nearly identical AUC's for the blood for both routes of administration were obtained. Assuming that the radiation dose to the blood reflects the radiation dose to the red marrow, this suggests that the administered activity given intraperitoneally equals the dose that can be administered intravenously. On the other hand, however, due to the high blood levels at early time points after i.v. administration, the dose rate to the bone marrow in the first hours after i.v. injection is higher than that after i.p. injection. Therefore, the MTD might be somewhat higher after i.p. administration.

In the therapy studies, abdominal circumference was used as a measure of i.p. tumor growth in order to predict therapeutic efficacy. Mice were monitored by one observer, to exclude inter-observer variability. I.p. tumor weight at dissection was similar in the groups, indicating that the increase in abdominal circumference correlated well with i.p. tumor load and can be used to estimate therapeutic efficacy. Results indicated that even the lowest dose of ^{131}I-labeled MN-14 (62.5 μCi) resulted in a small yet significant delay in tumor outgrowth, as compared to the control group that received unlabeled MN-14. All mice, however, eventually developed macroscopic or microscopic tumor.
The mean beta-emission of 192 keV of ^{131}I appears to be suited for the treatment of tumor nodules with a diameter of a few millimeters. Therapeutic efficacy in the current model might be improved using other radionuclides, such as ^{186}Re or ^{177}Lu. Theoretically, ^{90}Y, because of its high mean beta-emission of 935 keV, seems to be a less suitable radionuclide for this setting. Esteban et al.[34] studied the effects of 90Y-based radioimmunotherapy using an anti-CEA MAb in a similar model of small volume peritoneal LS174T carcinomatosis. Although inhibition of tumor growth proved to be significantly related to the activity dose administered, residual viable tumor growth was still found on histological examination of mice five weeks after receiving 120 μCi. In future experiments, the therapeutic efficacy of the above-mentioned radionuclides will be compared in the current model.
Finally, the observed dose-response relationship in the present study may have some useful implications. Firstly, at equitoxic activity dose levels lower than the MTD, the therapeutic efficacy of different radionuclides can be compared in a shorter period of time. Secondly, since there have been various reports on the use of radiosensitizers to enhance radioimmunotherapeutic efficacy, possible synergistic effects of chemotherapy combined with radioimmunotherapy in the current model can be studied at doses below MTD.
In summary, the radioiodinated MN-14 antibody accumulated preferentially in i.p. LS174T tumor nodules both after i.p. and i.v. administration. I.p. radioimmunotherapy using ^{131}I-labeled MN-14 significantly delayed the growth of peritoneal LS174T metastases, even at relatively low activity doses, which makes this model attractive for further investigation of the application of radioimmunotherapy for the treatment of early-stage PC.

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4

Biodistribution and therapeutic efficacy of $^{125/131}$I-, ^{186}Re-, $^{88/90}$Y- or ^{177}Lu-labeled anti-CEA monoclonal antibody MN-14 in mice with small peritoneal metastases of colorectal origin

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Radioimmunotherapy, using radiolabeled monoclonal antibodies (MAbs) against tumor-associated antigens, has not fulfilled its promise in solid cancers as it has done in hematological malignancies. Besides limited radiosensitivity of carcinomas as compared to hematological malignancies, solid tumors are generally characterized by a limited vascular supply, heterogeneous uptake of the antibody in the tumor, and elevated interstitial pressure in combination with a relatively long transport distance in the interstitium.[1] Radioimmunotherapy is therefore considered to be more suitable for the treatment of microscopic or minimal residual disease, allowing the radiolabeled MAbs to achieve uptake in tumors high enough to result in tumoricidal radiation doses.

An important issue in radioimmunotherapy is the selection of the radionuclide. Beta-emitting isotopes, such as ^{131}I and ^{90}Y, are the most commonly used radionuclides in radioimmunotherapy. ^{186}Re and ^{177}Lu are beta-emitting radionuclides that have been considered for radioimmunotherapy more recently. The physical characteristics of the four mentioned radionuclides, however, differ significantly with respect to half-life, the presence of gamma-radiation, the energy of the beta-emission and consequently the maximum penetration depth of the beta-particles in tissue, as summarized in Table 1.

We have previously characterized an experimental model of small peritoneal metastases using the human colon carcinoma cell line LS174T.[2] In this model radioimmunotherapy using ^{131}I-labeled MN-14 was very effective in delaying the development of peritoneal carcinomatosis, even at relatively low activity doses. Therapeutic efficacy in this model might be improved using other radionuclides with more favorable characteristics for radioimmunotherapy.

In the present study experiments were performed that aimed to select the most suitable radionuclide for radioimmunotherapy of small peritoneal metastases of colorectal origin. For this purpose, first a series of experiments was carried out investigating the biodistribution of $^{131/125}$I-, ^{186}Re- and ^{88}Y-labeled MN-14 in nude mice with small intraperitoneal (i.p.) xenografts of colon cancer. Then the therapeutic efficacy of MN-14 labeled with either ^{131}I, ^{186}Re, ^{90}Y or ^{177}Lu was assessed and correlated with the results of the biodistribution studies.

Material and methods

Animal model of small peritoneal metastases

Male nude BALB/c mice (Charles River Laboratories, Germany), 8-9 weeks old, weighing 20-25 grams were used in the experiments. Mice were accustomed to laboratory conditions for at least one week before experimental use and were housed under nonsterile standard conditions (temperature 20–24°C; relative humidity 50–60%;

Table 1. Physical characteristics of most used beta-emitters in radioimmunotherapy

Radionuclide	Half-life (days)	Max. ß-energy (MeV)	Max. penetration depth (mm)	γ-Emission* (keV)
^{131}I	8.0	0.606	3.0	364 (82), 637 (6.5)
^{90}Y	2.7	2.28	12.0	none
^{186}Re	3.8	1.08	5.4	137 (9.5)
^{177}Lu	6.7	0.497	2.5	208 (11), 113 (7)

**Numbers in parentheses are percentages*

12 h light/12 h dark) in filter-topped cages (five mice per cage), with free access to animal chow (Snif Voer, Soest, The Netherlands) and water. Peritoneal metastases were induced as described previously.[2] In brief, mice were inoculated intraperitoneally with 1.0 x 10^6 LS174T cells (CCL 188, American Type Culture Collection, Rockville, MD), suspended in 500 µl of RPMI-1640 medium in a 2.5 mL syringe using a 23-gauge needle. In this model, the first macroscopic tumor nodules are seen seven to ten days thereafter, whereas bulky peritoneal carcinomatosis develops three to five weeks after tumor cell inoculation. All experiments were approved by the institutional Animal Welfare Committee of the University Medical Center Nijmegen and conducted in accordance with the principles laid out by the revised Dutch Act on Animal Experimentation (1997).

Monoclonal antibody

The murine MN-14 MAb is a high-affinity (K_a = 10^9 M^{-1}) class-III anti-CEA IgG_1 antibody, produced by a hybridoma cell line culture, kindly provided by Immunomedics, Inc. (Morris Plains, New Jersey, USA).[3] The antibodies were purified by protein A chromatography, as described previously.[4] Purity was checked by fast protein liquid chromatography (FPLC) on a Phenomenex Biosep 3000 column, eluted with phosphate buffered saline (PBS, pH 7.2, 1 mL/min).

Radioiodination

Antibodies were radioiodinated with ^{125}I or ^{131}I (Amersham, Den Bosch, The Netherlands and MDS Nordion, Fleurus, Belgium, respectively) using the iodogen-method (1,3,4,6-tetrachloro-3α,6α-diphenyl-glycoluril; Pierce, Rockford, IL).[5] Briefly, antibodies (1.0 – 1.5 mg) and ^{125}I or ^{131}I were incubated at room temperature in 85 µl of PBS (0.10 M, pH 7.4) in a glass vial, coated with 50-100 µg iodogen. After ten minutes, the reaction was stopped by adding 100 µl of a saturated tyrosine solution. The reaction mixture was subsequently eluted on a PD-10 column (Amersham Biosciences, Uppsala, Sweden), eluted with PBS, 0.5% bovine serum albumin (BSA). Labeling efficiency of all radioiodination reactions exceeded 90%. In the biodistribution study specific activity of ^{125}I- and ^{131}I-MN-14 was 18.5 and 37 kBq/µg. Specific activity of ^{131}I-MN-14 in the therapy study was 0.46 MBq/µg.

^{186}Re-labeling

$^{186}ReO_4^-$ (specific activity 65 GBq/mg) was obtained from Tyco Mallinckrodt Medical BV (Petten, The Netherlands). The antibodies were labeled with ^{186}Re using S-benzoyl-mercaptoacetyltriglyceride (S-benzoyl-MAG3) as chelator, as described by Visser et al.[6] Briefly, 180 µg MAG3 (1.0 mg/ml) was incubated with 150 µL 1.0 M Na_2CO_3, 150 µL Na_2SO_3 (100 mg/ml) and 696 MBq $^{186}ReO_4^-$ (100 µL) in a boiling water bath (10 min). The solvent was then evaporated after which the solid phase was incubated for another 15 minutes. Following derivatization of ^{186}Re-MAG3 with 2,3,5,6-tetrafluorophenol (TFP, 100 mg/ml in MeCN/H_2O 9:1), the derivatized ^{186}Re-MAG3-ester was purified on a Sep-Pak C18 cartridge (Waters, Milford, MA) and subsequently reacted with 400 µg of a concentrated MN-14 solution at pH 9.5, resulting in a mean number of 4.1 MAG3 groups per IgG molecule. The conjugated ^{186}Re-MAG3-MN-14 (further referred to as ^{186}Re-MN-14) was purified on a PD-10 column. The overall labeling efficiency of the ^{186}Re-labeling procedures performed in the biodistribution and therapy studies was 36% and 18%, resulting in specific activities of 0.12 MBq/µg and 0.33 MBq/µg, respectively.

$^{88/90}$Y- and ^{177}Lu-labeling

All conjugation and labeling procedures were performed under strict metal-free conditions. To allow labeling of the antibodies with ^{88}Y, ^{90}Y and ^{177}Lu, MN-14 was conjugated with isothiocyanatobenzyl-diethylenetriaminepentaacetic acid (ITC-DTPA, Macrocyclics, Dallas, TX). Briefly, ITC-DTPA was conjugated to MN-14 in a 0.1 M $NaHCO_3$ buffer, pH 8.2 using a 100-fold (ITC-DTPA) molar excess as described by Ruegg et al.[7] with minor modifications (conjugation period of one hour at room temperature). The DTPA-MAb MN-14 conjugate was purified by extensive dialysis against 0.1 M ammonium acetate buffer (pH 5.0). The number of DTPA ligands per antibody molecule was determined according to the method described by Hnatowich et al.[8] The purified

DTPA-MN-14 conjugate (DTPA/MN-14 ratio 2.5/1, 0.8 mg/mL) was incubated with ^{88}Y (Isotope Products Europe Blaseg, Waldburg, Germany), ^{90}Y (Perkin Elmer, Brussels, Belgium) or ^{177}Lu (University of Missouri, Research Reactor, Columbia, MO, USA) in 0.1 M ammonium acetate buffer, pH 5.4 at room temperature (20 minutes). Specific activities of the ^{88}Y-DTPA-MN-14, ^{90}Y-DTPA-MN-14 and ^{177}Lu-DTPA-MN-14 preparations (further referred to as ^{88}Y-MN-14, ^{90}Y-MN-14 and ^{177}Lu-MN-14) were 48.1, 159 and 418 kBq/µg respectively.

Quality control of the radiolabeled preparations

All radiolabeled MN-14 preparations were purified by gel filtration on a PD-10 column and eluted with PBS supplemented with 0.5 % BSA. For all preparations the amount of free radiolabel was determined by instant thin layer chromatography (ITLC) using ITLC silica gel strips (Gelman Sciences, Inc., Ann Arbor, MI, USA) using 0.1 M citrate buffer (pH 6.0) as the mobile phase. Radiochemical purity of all radiolabeled antibody preparations used in the studies exceeded 96%.
The immunoreactive fraction (IRF) at infinitive antigen excess of the radiolabeled MN-14 preparations except ^{90}Y-MN-14 was determined on freshly trypsinized LS174T cells essentially as described by Lindmo et al.[9] with minor modifications. Briefly, a fixed amount of labeled antibody (10,000 cpm) was incubated with increasing concentrations of LS174T tumor cells (1.2 x 10^6 – 20 x 10^6 cells/mL) in 0.5 mL binding buffer (RPMI medium containing 0.5% BSA and 0.05% NaN_3). A duplicate of the lowest cell concentration was incubated in the presence of an excess unlabeled antibody to correct for non-specific binding. After six hours of incubation at 37°C, the cells were washed and activity in the pellet was determined in a well-type gamma-counter. The inverse of the tumor cell bound fraction was plotted against the inverse of the cell concentration and the immunoreactive fraction (IRF) was calculated from the Y-axis intercept. The mean IRFs of the radiolabeled preparations used in the biodistribution and therapy studies were 89.3±6.5% and 77±6.1%, respectively. Labeled antibody preparations were administered within two hours after radiolabeling.

Biodistribution studies after i.p. and i.v. administration

To assess the effect of both the route of administration and the radiolabel on the biodistribution of radiolabeled MN-14, mice were inoculated intraperitoneally with 1.0 x 10^6 LS174T tumor cells suspended in RPMI-1640 (500 µl). Ten days later, mice received 1.22 MBq ^{186}Re-MN-14 intraperitoneally and 0.481 MBq ^{88}Y-MN-14 intravenously or vice versa (five mice per group). Mice were killed by O_2/CO_2-asphyxiation and dissected at 24, 48, 72, 96 or 192 hours after the administration of the radiolabeled antibody preparations (five mice per group). At dissection tumor, blood, liver, spleen, kidney, intestine, lung, muscle as well as the right femur were sampled, blotted dry, and weighed. Activity was measured in a shielded well-type gamma-counter (Wizard,

Pharmacia-LKB, Sweden). To correct for physical decay and to calculate uptake of the radiolabeled antibody in each sample as a fraction of the injected dose, aliquots of the injected dose were counted simultaneously. The results were expressed as percentage of the injected dose per gram tissue (%ID/g). Since we showed in a previous study that at protein doses exceeding 25 μg, the uptake of the radiolabeled MN-14 antibody in tumor tended to be lower,[2] the total protein dose of each preparation was adjusted to 10 μg per mouse (total: 20 μg per mouse). The results were compared with the previously reported data on the biodistribution of radioiodinated MN-14 after i.v. versus i.p. administration in the same model.[2]

Estimation of radiation dose to the tumor

The biodistribution data were used to calculate the areas-under-the-curve (AUC), corrected for physical decay. Subsequently, the data were processed using the MIRDOSE3 software program[10] (Oak Ridge associated Universities), in order to estimate the absorbed radiation dose to the tumor for ^{131}I-MN-14, ^{186}Re-MN-14, ^{90}Y-MN-14 and ^{177}Lu-MN-14 at 50% of their maximal tolerated activity doses (MTD). For this purpose, it was assumed that the biodistribution of both ^{90}Y-MN-14 and ^{177}Lu-MN-14 would have been similar to that of ^{88}Y-MN-14.

Radioimmunotherapy studies

Ten days after i.p. tumor cell inoculation, groups of ten mice received i.p. injections of ^{131}I-MN-14 (9.25 MBq), ^{186}Re-MN-14, (9.25 MBq), ^{90}Y-MN-14 (3.15 MBq), ^{177}Lu-MN-14 (8.33 MBq/mouse) or unlabeled MN-14 (control). The activity doses represented equitoxic dose levels of the respective radionuclides, i.e., equal to 50% of the respective MTDs. The MTD of each antibody-bound radionuclide after i.p. administration was defined as the activity dose below the lowest dose level that resulted in either death of any animal in groups of five animals, or body weight loss of more than 20%, and was empirically determined as described previously.[11] The MTDs for ^{131}I-MN-14, ^{186}Re-MN-14, ^{90}Y-MN-14 and ^{177}Lu-MN-14 after i.p. administration were 18.5 MBq, 18.5 MBq, 6.29 MBq and 16.65 MBq, respectively. Preparations were administered in 400 μl PBS, 0.5% BSA. Protein dose was adjusted to 20 μg by adding unlabeled MN-14 to a fraction of the primary radioimmunoconjugate preparations. Due to a lower labeling efficiency than anticipated, protein dose of the ^{186}Re-MN-14 preparation was 28 μg. Mice were monitored daily and body weight and abdominal circumference was measured twice per week (MJK), as described previously.[2] Besides death, humane endpoints were defined as a decrease of body weight of 20% or more or an abdominal circumference increase of 10% or more due to i.p. tumor growth as compared to the abdominal circumference measured on the day of tumor inoculation. When either one of these criteria was met, mice were killed by O_2/CO_2 asphyxiation and cervical dislocation. All i.p. tumor deposits were meticulously dissected and weighed. The experiment was

terminated at 142 days after tumor cell inoculation when the remaining mice were euthanized and dissected. The abdominal cavity was conscientiously inspected. Liver, spleen, lungs, pancreas, greater omentum and the diaphragm were removed for routine histopathological H&E-staining and immunohistochemical staining using a rabbit-anti-human anti-CEA polyclonal antibody (A 0115, DakoCytomation, Glostrup, Denmark).[12]

Statistical analysis

Statistical analysis was performed by means of the GraphPad InStat 3.00 software (GraphPad Software, San Diego, CA). Single comparisons were analyzed using the two-tailed, Welch corrected, unpaired t-test or the non-parametric, two-tailed Mann Whitney^{U} test. In the biodistribution studies, uptake in tissues and blood levels were compared using the one-way ANOVA test. Bonferroni correction for multiple testing was applied. In the therapy studies, survival curves were compared using the Log-rank test. For all tests, the level of significance was set at a P value of <0.05.

Results

Biodistribution of radioiodinated MN-14

The biodistribution of radioiodinated MN-14 in this model following i.p. or i.v. administration has been described previously.[2] In brief, from 24 hours onwards, the blood levels of the intraperitoneally injected ^{131}I-MN-14 and the intravenously injected ^{125}I-MN-14 were similar. In the first 24 hours after administration, the i.p. route resulted in higher uptake in the tumor than the i.v. route. From 48 hours p.i. onwards, tumor uptake was similar for both routes of administration (approximately 50 %ID/g). When corrected for physical decay of ^{131}I, the AUCs for blood levels were similar for both routes of administration (0.56 versus 0.58 h x MBq/g), whereas the AUC for tumor after i.p. administration was somewhat higher (2.46 versus 1.96 h x MBq/g).

Comparison of biodistribution of radioiodinated MN-14, ^{186}Re- and ^{88}Y-labeled MN-14 after i.p. or i.v. administration

Mean tumor weight of the mice that received radioiodinated MN-14 was comparable to that of the mice that received ^{186}Re-MN-14 or ^{88}Y-MN-14 (19.5 ± 24.2 mg versus 16.1 ± 16.6 mg; p=0.50). The blood levels of radioiodinated, ^{186}Re-, or ^{88}Y-labeled MN-14 after i.p. and i.v. administration are depicted in Figure 1a-b. From 24 hours p.i. onwards, blood levels for all radioimmunoconjugates were similar for both routes of administration. The tissue distribution of ^{186}Re-MN-14 and ^{88}Y-MN-14 after both routes

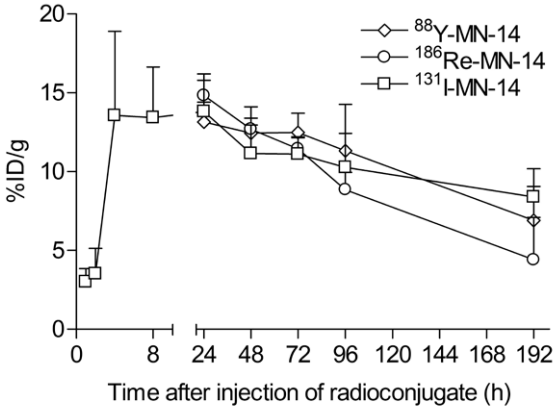

Figure 1a
Blood levels after intraperitoneal administration of ^{131}I-, ^{186}Re- and ^{88}Y-labeled MN-14. Values are given as means ± standard deviation (five mice per group).

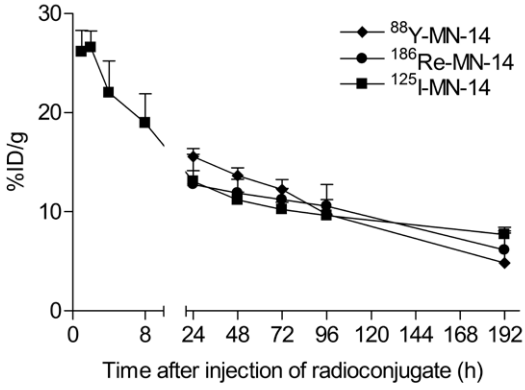

Figure 1b
Blood levels after intravenous administration of ^{125}I-, ^{186}Re- and ^{88}Y-labeled MN-14. Values are given as means ± standard deviation (five mice per group).

of administration is summarized in Tables 2 and 3. At all time points, uptake in liver and spleen of ^{88}Y-MN-14 was higher than that of radioiodinated MN-14 and ^{186}Re-MN-14 after both routes of administration. Uptake in liver of ^{88}Y-MN-14 remained higher after i.v. administration than after i.p. administration throughout the experiment, whereas uptake in spleen was similar at all time points. From 48 hours p.i. onwards, uptake of ^{88}Y-MN-14 in bone represented by the femur was significantly higher than that of ^{186}Re-MN-14. However, maximum uptake of ^{88}Y-MN-14 in bone was very low (2.6 ± 0.3% ID/g, 72 hours after i.v. administration). Uptake in other normal tissues was similar for all radioimmunoconjugates after both routes of administration.
In Figure 2a-b the uptake in tumor of ^{131}I-MN-14, ^{186}Re-MN-14 and ^{88}Y-MN-14 after i.p. and i.v. administration is depicted. Uptake in tumor of ^{88}Y-MN14 was higher than that of ^{186}Re-MN-14 or radioiodinated MN-14 at all time points, except for 24 hours after i.v. administration. Maximum uptake after i.p. administration of ^{131}I-MN-14, ^{186}Re-

Table 2. Tissue distribution of intraperitoneally and intravenously administered ^{186}Re-MN-14 in nude mice bearing intraperitoneal LS174T colon cancer xenografts

Organ	Time post-injection radioconjugate (hours)				
	24	48	72	96	192
Intraperitoneally administered					
Tumor	29.9 ± 7.3	77.8 ± 31.4	83.4 ± 18.5	66.0 ± 5.4	50.3 ± 29.2
Blood	14.8 ± 1.0	12.7 ± 0.7	11.4 ± 0.8	8.9 ± 1.1	4.4 ± 2.7
Muscle	1.24 ± 0.4	1.2 ± 0.1	1.2 ± 0.1	1.6 ± 1.4	0.5 ± 0.2
Lung	8.3 ± 1.2	7.0 ± 0.8	8.4 ± 1.5	6.6 ± 1.1	3.8 ± 2.2
Spleen	3.8 ± 0.7	3.5 ± 0.4	3.2 ± 0.4	2.3 ± 0.5	1.6 ± 0.6
Kidney	4.3 ± 0.2	3.7 ± 0.2	3.4± 0.1	3.2 ± 0.4	1.7 ± 0.7
Liver	5.2 ± 0.7	4.8 ± 0.3	4.6 ± 0.5	3.9 ± 0.5	2.0 ± 0.7
Intestine	3.5 ± 0.8	1.8 ± 0.3	2.1 ± 0.4	1.5 ± 0.5	0.7 ± 0.3
Femur	0.8 ± 0.1	1.1 ± 0.2	1.2 ± 0.2	0.7 ± 0.2	0.6 ± 0.2
Intravenously administered					
Tumor	58.1 ± 18.4	50.3 ± 21.1	68.2 ± 10.7	65.1 ± 14.2	67.8 ± 42.3
Blood	12.8 ± 3.0	11.9 ± 1.36	11.2 ± 1.1	10.6 ± 2.2	6.14 ± 1.9
Muscle	0.9 ± 0.2	1.2 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	0.7 ± 0.4
Lung	7.8 ± 2.4	10.5 ± 1.1	8.5 ± 0.8	9.6 ± 1.3	5.8 ± 1.8
Spleen	3.8 ± 1.1	3.2 ± 0.3	2.8 ± 0.3	2.5 ± 0.2	1.9 ± 0.4
Kidney	3.8 ± 0.8	3.8 ± 0.3	3.3 ± 0.4	2.9 ± 0.3	1.5 ± 1.0
Liver	5.4 ± 1.2	4.7 ± 0.3	4.5 ± 0.8	3.5 ± 0.5	1.6 ± 1.1
Intestine	2.8 ± 0.9	2.2 ± 0.3	1.7 ± 0.1	1.4 ± 0.1	1.2 ± 0.2
Femur	0.9 ± 0.4	0.8 ± 0.4	1.0 ± 0.2	0.5 ± 0.1	0.5 ± 0.1

Values are given as means ± standard deviation (five mice per group)

Table 3. Tissue distribution of intraperitoneally and intravenously administered ^{88}Y-MN-14 in nude mice bearing intraperitoneal LS174T colon cancer xenografts

Organ	Time post-injection radioconjugate (hours)				
	24	48	72	96	192
Intraperitoneally administered					
Tumor	90.8 ± 13.4	83.5 ± 29.0	139.5 ± 22.9	128.5 ± 26.5	148.1 ± 89.4
Blood	13.2 ± 1.3	12.4 ± 1.7	12.5 ± 1.2	11.3 ± 3.0	6.9 ± 2.2
Muscle	0.9 ± 0.1	1.4 ± 0.2	1.2 ± 0.2	0.9 ± 0.1	1.0 ± 0.5
Lung	7.9 ± 1.2	11.3 ± 1.5	10.0 ± 1.0	10.5 ± 2.0	7.2 ± 2.3
Spleen	4.9 ± 0.8	5.2 ± 0.5	5.8 ± 0.8	5.9 ± 0.9	7.9 ± 1.3
Kidney	4.0 ± 0.3	4.2 ± 0.4	3.9 ± 0.4	3.3 ± 0.6	1.6 ± 1.5
Liver	6.6 ± 0.6	6.7 ± 1.1	7.4 ± 1.3	6.8 ± 1.3	4.8 ± 3.1
Intestine	2.9 ± 0.7	2.5 ± 0.5	2.0 ± 0.1	1.7 ± 0.3	1.7 ± 0.3
Femur	0.9 ± 0.2	1.3 ± 0.5	2.0 ± 0.4	1.3 ± 0.2	2.4 ± 0.7
Intravenously administered					
Tumor	27.4 ± 9.4	105.3 ± 50.5	130.5 ± 49.9	115.1 ± 5.4	104.3 ± 55.9
Blood	15.6 ± 0.8	13.6 ± 0.8	12.2 ± 1.0	9.8 ± 1.5	4.9 ± 3.0
Muscle	1.0 ± 0.2	1.2 ± 0.1	1.3 ± 0.2	2.3 ± 2.6	0.6 ± 0.3
Lung	8.8 ± 1.2	8.0 ± 0.9	9.4 ± 1.9	8.0 ± 1.4	4.7 ± 2.4
Spleen	5.1 ± 0.9	6.8 ± 0.4	6.4 ± 0.9	5.8 ± 0.6	7.1 ± 0.8
Kidney	4.4 ± 0.2	4.1 ± 0.2	3.9 ± 0.3	3.8 ± 0.5	2.3 ± 0.9
Liver	9.5 ± 0.8	9.8 ± 0.6	9.4 ± 0.9	10.1 ± 1.9	8.9 ± 2.2
Intestine	3.6 ± 0.8	2.0 ± 0.4	2.5 ± 0.4	1.9 ± 0.7	0.9 ± 0.3
Femur	0.6 ± 0.3	2.0 ± 0.5	2.6 ± 0.3	1.5 ± 0.2	2.1 ± 0.8

Values are given as means ± standard deviation (five mice per group)

MN-14 and ^{88}Y-MN14 in tumor was 58.5 ± 6.8% ID/g (24 hours p.i.), 83.4 ± 18.5% ID/g (72 hours p.i.) and 148.1 ± 89.4% ID/g (192 hours p.i.), respectively.
The tumor-to-blood ratios of the various radioimmunoconjugates after i.p. and i.v. administration are shown in Figure 3a-b. From 24 hours p.i. onwards, the tumor-to-blood ratio of radioiodinated MN-14 remained relatively stable (between 4.0 and 6.0), whereas tumor-to-blood ratio of ^{186}Re-MN-14 steadily increased to 12.0 at 192 hours p.i. Tumor-to-blood ratios of ^{88}Y-MN-14 were higher than those of both radioiodinated MN-14 and ^{186}Re-MN-14 at every time point. Maximum tumor-to-blood ratio of ^{88}Y-MN-14 was reached at 192 hours p.i. (24.7 ± 11.0 after i.v. administration).

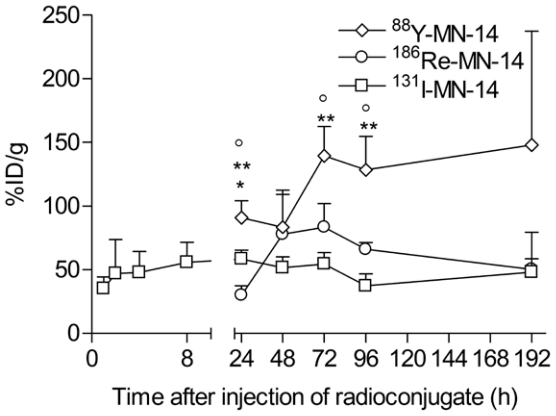

Figure 2a
*Uptake of ^{131}I-, ^{186}Re- and ^{88}Y-labeled MN-14 in peritoneal LS174T tumor xenografts after intraperitoneal administration. Values are given as means ± standard deviation (five mice per group). ** Significant difference between ^{88}Y and ^{186}Re; * Significant difference between ^{186}Re and ^{131}I; * Significant difference between ^{88}Y and ^{131}I (one-way ANOVA test with Bonferroni post-correction).*

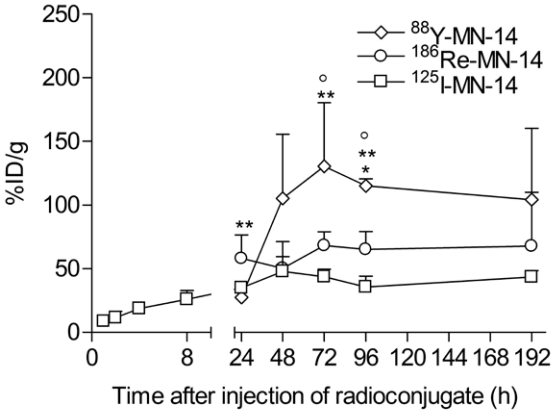

Figure 2b
*Uptake of ^{125}I-, ^{186}Re- and ^{88}Y-labeled MN-14 in peritoneal LS174T tumor xenografts after intravenous administration. Values are given as means ± standard deviation (five mice per group). ** Significant difference between ^{88}Y and ^{186}Re; * Significant difference between ^{186}Re and ^{125}I; * Significant difference between ^{88}Y and ^{125}I (one-way ANOVA test with Bonferroni post-correction).*

Tumor absorbed radiation dose

Dosimetric analysis of the biodistribution data (summarized in Figure 2a and Table 2 and 3) using the MIRD methodology indicated that i.p. administration of equitoxic activity doses (50% of MTD) of ^{131}I-MN-14 (9.25 MBq), ^{186}Re-MN-14, (9.25 MBq), ^{90}Y-MN-14 (3.15 MBq), or ^{177}Lu-MN-14 (8.33 MBq/mouse) would result in absorbed radiation doses to tumors of 150 Gy, 100 Gy, 45 Gy and 200 Gy, respectively. In these calculations it was assumed that the weight of the tumor nodules was 10 mg, corresponding to a diameter of 2.6 mm.

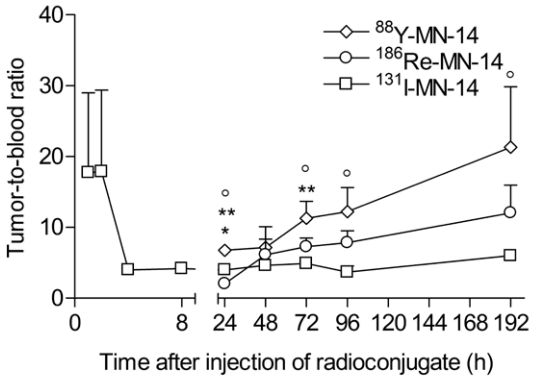

Figure 3a
*Tumor-to-blood ratios after intraperitoneal administration of ^{131}I-, ^{186}Re- and ^{88}Y-labeled MN-14 in mice bearing peritoneal LS174T tumor xenografts. Values are given as means ± standard deviation (five mice per group). ** Significant difference between ^{88}Y and ^{186}Re; * Significant difference between ^{186}Re and ^{131}I; * Significant difference between ^{88}Y and 131I (one-way ANOVA test with Bonferroni post-correction).*

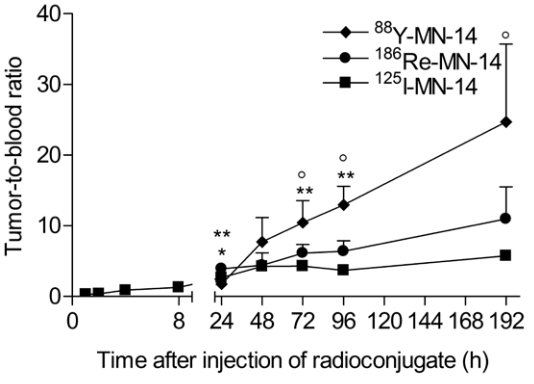

Figure 3b
*Tumor-to-blood ratios after intravenous administration of ^{125}I-, ^{186}Re- and ^{88}Y-labeled MN-14 in mice bearing peritoneal LS174T tumor xenografts. Values are given as means ± standard deviation (five mice per group). ** Significant difference between ^{88}Y and ^{186}Re; * Significant difference between ^{186}Re and ^{125}I; ± Significant difference between ^{88}Y and ^{125}I (one-way ANOVA test with Bonferroni post-correction).*

Radioimmunotherapy

Three mice developed subcutaneous tumors at the site of tumor cell inoculation and were therefore excluded from the analysis. Thirty-three mice were killed when their abdominal circumference had increased by 10% due to i.p. tumor growth. Mean tumor weight in these mice was 2.23 ± 1.10 gram. Furthermore, five mice were killed due to 20% loss of body weight and three additional mice because of poor clinical condition without weight loss being 20% or abdominal circumference increase reaching 10%. Mean tumor weight of the latter eight mice was 1.62 ± 0.92 gram, which was not statistically different from the tumor load found in the 33 mice mentioned above (p=0.25). The survival curves of the different treatment groups are shown in Figure 4. Median survival of the control mice that received unlabeled MN-14 was 42 days (range 32-49). Median survival of the mice treated with equitoxic activity doses of ^{186}Re-MN-14 (9.25 MBq), ^{90}Y-MN-14 (3.15 MBq), ^{131}I-MN-14 (9.25 MBq) or ^{177}Lu-MN-14 (8.33 MBq/mouse) was 72 days (range 46-77; p=0.0002), 82 days (46-142, p=0.0001), 100 days (58-142, p<0.0001) and 136 days (range 56-142; p<0.0001), respectively (p vs. unlabeled MN-14 control group, Log-rank test). The p-values of the differences between the survival curves of the various treatment groups are shown in Table 4.

At the end of the experiment (142 days after tumor cell inoculation), there were nine mice (^{177}Lu-MN-14, n=5; ^{131}I-MN-14, n=3; ^{90}Y-MN-14, n=1) without any signs of i.p. tumor growth. At dissection one mouse treated with ^{177}Lu-MN-14 had some tumor growth (total i.p. tumor load, 0.65 g), whereas in the remaining eight mice there was no evidence of disease. On histopathological examination of the diaphragm, greater omentum, pancreas, liver, spleen and lungs, no residual disease was found in any of these mice.

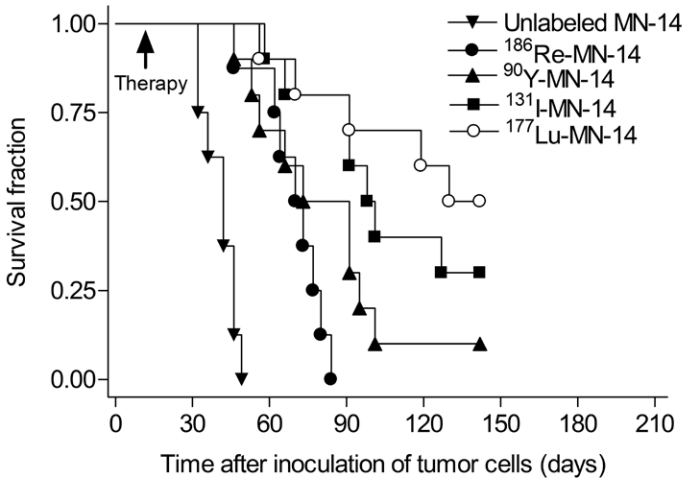

Figure 4
Survival curves of mice bearing peritoneal LS174T tumor xenografts after i.p. administration of ^{131}I-MN-14 (9.25 MBq), ^{186}Re-MN-14 (9.25 MBq), ^{90}Y-MN-14 (3.15 MBq), ^{177}Lu-MN-14 (8.33 MBq per mouse) or unlabeled MN-14 (eight to ten mice per group). P-values following comparison of the survival curves of the various treatment groups are given in Table 4.

Table 4. P-values following comparison of survival curves of mice treated with unlabeled MN-14 or MN-14 labeled with equitoxic activity doses of ^{186}Re, ^{131}I, ^{90}Y or ^{177}Lu

Radionuclide (median survival)	Unlabeled MN-14	^{186}Re-MN-14	^{90}Y-MN-14	^{131}I-MN-14	^{177}Lu-MN-14
Unlabeled MN-14 (42)	_	0.0002	<0.0001	<0.0001	<0.0001
^{186}Re-MN-14 (72)	0.0002	_	0.11	0.0014	0.0012
^{90}Y-MN-14 (82)	<0.0001	0.11	_	0.10	0.02
^{131}I-MN-14 (100)	<0.0001	0.0014	0.10	_	0.36
^{177}Lu-MN-14 (136)	<0.0001	0.0012	0.02	0.36	_

P-values were determined by use of the Log-rank test

Discussion

The primary aim of this study was to select the most suitable radionuclide for radioimmunotherapy for the treatment of small peritoneal metastases of colorectal origin. Radioimmunotherapy using ^{177}Lu-MN-14 resulted in the best median survival of 136 days, which was significantly better as compared to that after treatment with ^{186}Re-MN-14 (72 days) or ^{90}Y-MN-14 (82 days) but which did not differ significantly from survival following treatment with ^{131}I-MN-14 (100 days). No residual tumor was found by histopathological examination 142 days after tumor cell inoculation in four out of ten mice treated with ^{177}Lu-MN-14, three out of ten mice treated with ^{131}I-MN-14 and one out of ten mice treated with ^{90}Y-MN-14, which were considered cured at the end of the study.

In the biodistribution studies, ^{88}Y-labeled MN-14 resulted in a much higher uptake in tumor than either radioiodinated MN-14 or ^{186}Re-MN-14. The higher uptake of ^{88}Y-MN-14 in the tumor nodules probably reflects a longer tumor residence time of ^{88}Y as compared to that of ^{131}I or ^{186}Re, which ensues from differences between the various radiolabels in intratumoral catabolization.[13] Although anti-CEA monoclonal antibodies, such as MN-14, that bind to CEA-epitopes on the tumor cell surface are internalized only slowly and to a limited extent[14, 15] intratumoral catabolism of antibodies has been shown to be significant not only for rapidly internalizing antibodies, but also for antibodies binding

to the cell surface.[16] After internalization by the cancer cell, radiolabeled antibodies are enzymatically degraded and metabolized in the lysosomes.[17, 18] After intralysosomal metabolization of monoclonal antibodies that are radioiodinated by conventional methods, the radioiodinated tyrosine residues are excreted, thereby reducing the residence times of the radioiodine label in the tumor.[13] After catabolization of MAbs labeled with $^{88/90}$Y- or ^{177}Lu-DTPA, the catabolic products are the radiolabeled chelators bound to amino acids, such as lysine (e.g. ^{88}Y-DTPA-lysine).[19, 20] Whereas radioiodinated tyrosine is excreted by the cell, the $^{88/90}$Y- or ^{177}Lu-DTPA-lysine metabolites are trapped within the lysosomes. Furthermore, since antibodies are metabolized by the liver and spleen, residualization of the ^{88}Y radiolabel may also explain the higher uptake in these organs as compared to that of $^{125/131}$I or ^{186}Re. To date, the fate and processing of ^{186}Re-MAG3-labeled antibodies bound to the surface of tumor cells has not been elucidated. Various studies, however, have shown that ^{186}Re is not retained in the cell after intracellular catabolization.[17, 21] The higher uptake of ^{186}Re-MN-14 in tumor and the higher tumor-to-blood ratios as compared to that of radioiodinated MN-14 in our studies, however, suggest that the catabolic product of ^{186}Re-MAG3-MN-14 (presumably ^{186}Re-MAG3-lysine) may be released from the cell at a slower rate than radioiodine.

In the therapy studies, the administered activity doses of the different radioimmunoconjugates represented 50% of the MTDs for the various radionuclides. The therapeutic efficacy of ^{90}Y-MN-14 was much lower than that of ^{177}Lu-MN-14, but did not differ significantly from that of ^{131}I-MN-14 nor from that of ^{186}Re-MN-14. Because of its high mean beta-emission of 935 keV and consequently a relatively high tissue penetration depth (maximal range 12 mm), irradiation of the small peritoneal metastases of only a few millimeters in this model is inefficient, since approximately 70% of the radiation energy is deposited outside the tumor xenografts. Indeed, dosimetric analysis of the biodistribution data indicated that the tumor-absorbed radiation dose for ^{90}Y-MN-14 was much lower as compared with the other radiolabels. Esteban et al.[22, 23] studied the effects of ^{90}Y-based radioimmunotherapy using the anti-CEA MAb ZCE025 in a similar model of small volume peritoneal LS174T carcinomatosis. In this study a clear dose-response effect was observed, although residual viable tumor growth was still found on histological examination of mice five weeks after receiving 4.44 MBq. Furthermore, Sharkey et al.[24] showed that in a mouse model of micrometastatic colon carcinoma in the lungs, radioimmunotherapy with ^{131}I was more effective than ^{90}Y. Therefore, ^{90}Y seems to be more appropriate for radioimmunotherapeutic application in larger tumors, which is in line with the findings of other investigators.[15, 25]

Although the dosimetric analysis correctly predicted ^{177}Lu to be more efficacious than ^{186}Re and ^{90}Y, the apparently higher tumor absorbed dose for ^{186}Re as compared to ^{90}Y did not result in an improved survival. This discrepancy may be due to inaccuracies of the dosimetric analysis, since blood, bone marrow, liver and spleen were not included as source organs in this analysis. It may very well be feasible that the relatively high uptake of ^{90}Y-MN-14 in the liver and spleen (approximately 7 %ID/g after i.p. administration) in combination with its high-energy beta-emission (max. range 12 mm) may

have contributed to the sterilization of the small peritoneal metastases in the upper abdomen. Another explanation for the observed discrepancy between the survival and the tumor absorbed radiation dose may be failure of the animal model to reveal small differences in therapeutic efficacy.
Treatment of mice with ^{177}Lu-MN-14 resulted in the highest median survival of 136 days, which, however, did not differ significantly from that after treatment with ^{131}I-MN-14 (100 days; p=0.36). Given the longer intratumoral residence times of ^{177}Lu as compared to that of ^{131}I or ^{186}Re, this radionuclide, with its medium-energy beta (maximum penetration depth, 2.5 mm) and a half-life of almost a week, seems to be very well suited for the treatment of microscopic or small volume disease. From a clinical point of view, the radiophysical characteristics of ^{177}Lu may furthermore be more favorable as compared to those of ^{131}I or ^{90}Y. Firstly, due to its longer half-life of 6.7 days as compared to that of ^{90}Y (2.3 days), bone marrow toxicity may be less since less of the decay occurs in the early time period after administration when blood levels are relatively high and uptake in tumor is still low. Simultaneously, after intratumoral accumulation of the radiolabeled antibody, the tumor is irradiated over a prolonged period of time. Finally, the emission of low abundance, moderate-energy gamma rays of ^{177}Lu poses less radiation safety issues than those of ^{131}I for the patient's family and the health care personnel. In fact, promising results have been reported by Alvarez et al.[26], who treated 27 patients with chemotherapy-refractory ovarian cancer with i.p. radioimmunotherapy using ^{177}Lu-labeled CC-49 IgG antibody. Antitumor effects were noted even at lower dose levels, whereas patients with microscopic disease showed a prolonged disease-free survival compared to historical controls.

Conclusion

Uptake of ^{88}Y-MN-14 in small peritoneal LS174T xenografts was higher than that of ^{186}Re-MN-14 or ^{131}I-MN-14. At equitoxic activity doses, therapeutic efficacy of ^{177}Lu-MN-14 was better that of ^{186}Re-MN-14 and ^{90}Y-MN-14, but did not differ significantly from that of ^{131}I-MN-14, which is in line with the dosimetric analysis. The results of these studies indicate that ^{177}Lu and ^{131}I are the most suitable radionuclides for radioimmunotherapy of small peritoneal metastases and may be the best candidates for adjuvant treatment of patients at high risk for the development of i.p. relapse of colorectal cancer.

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5

Combination therapy using the cyclooxygenase-2 inhibitor parecoxib and radioimmunotherapy in nude mice with small peritoneal metastases of colonic origin

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Despite the promising results in hematological malignancies, the only modest therapeutic efficacy of radioimmunotherapy (RIT) using radiolabeled monoclonal antibodies (MAbs) in patients with solid cancers has tempered the initial enthusiasm with which this treatment modality was once greeted.[1] The targeting of intravenously administered radiolabeled antibodies to solid tumors is a relatively inefficient process due to various tumor-related factors, including a limited vascular supply, heterogeneous uptake of the antibody in the tumor, and elevated interstitial pressure in combination with a relatively long transport distance in the interstitium.[2] As a result, tumor uptake of radiolabeled MAbs and, consequently, the radiation doses delivered to solid lesions are in most cases too low to induce objective tumor responses. Several innovative approaches have been pursued with the aim of improving the efficacy of RIT. These include strategies to improve the localization and retention of radiolabeled MAbs in the tumor (e.g. using high-affinity MAbs[3,4]), strategies to accelerate the blood clearance of MAbs (e.g. using antibody fragments[5,6] or the pretargeting method[7]), and, strategies to increase the sensitivity of the tumor cells to radiation (e.g. by means of radiosensitizers[8-10]).

In the early nineteen-nineties, the inducible second isoform of cyclooxygenase (COX-2) was recognized to play a significant role in colorectal carcinogenesis. This observation was derived from epidemiological studies indicating that chronic users of non-steroid anti-inflammatory drugs (NSAIDs) had a 40-50% reduced risk of developing colorectal cancer (CRC).[11] The COX-2 enzyme catalyzes the conversion of arachidonic acid to prostaglandin derivatives and its expression is upregulated at sites of inflammation[12] and in various epithelial cancers, including 80-90% of human colon carcinomas.[13] To date, COX-2 has been acknowledged to be involved in various biological processes in epithelial and nonepithelial cancers, including the regulation of apoptosis,[14,15] tumor cell invasiveness and metastatic potential[16], tumor angiogenesis,[17] and protection against radiation damage.[18]

Because of the gastrointestinal and hematological toxicity of nonselective NSAIDs,[19] the potential of selective inhibitors of the COX-2 enzyme (so-called coxibs) to act as radiosensitizing agents has been subject of investigation in various preclinical and ongoing clinical studies. Coxibs were indeed shown to increase the sensitivity of tumor cells to external beam radiation in various experimental models of colon cancer,[20] breast cancer,[21] head and neck cancer,[20,22] lung cancer,[21,23] sarcoma,[24,25] and glioma[26] without increasing radiation-induced toxicity to normal tissue. To date, however, there have been no publications on the use of COX-2 inhibitors in combination with RIT.

We have characterized a nude mouse model for RIT of small volume peritoneal carcinomatosis of colorectal origin using the human colon carcinoma cell line LS174T.[27,28] RIT using the radiolabeled anti-CEA MAb MN-14 proved very effective in delaying the growth of intraperitoneal tumor xenografts, even at relatively low activity doses. The COX-2 enzyme is upregulated and actively mediates the production of prostaglandin E_2 (PGE_2) in the LS174T tumor cells.[29] PGE_2 has been shown to promote the growth

and metastatic potential of colon carcinoma cells in vitro and implicated as a protector against radiation damage.[18]
The novel, selective COX-2 inhibitor parecoxib is a prodrug, specifically designed for parenteral administration.[30] After systemic administration, parecoxib is rapidly converted to the COX-2 inhibitor valdecoxib by enzymatic hydrolysis in the liver.[31,32] Valdecoxib is one of the most potent and selective COX-2 inhibitors that have been developed to date.[33,34] Since parecoxib monotherapy has been reported to have antitumor effects in mouse models of breast cancer and colon cancer,[35,36] we hypothesized that co-administration of the COX-2 inhibitor parecoxib might sensitize the LS174T xenografts to radiation and improve the therapeutic efficacy of RIT in our model of small volume peritoneal carcinomatosis of colorectal origin.
To test this hypothesis, we designed a series of experiments that aimed to determine the efficacy of parecoxib monotherapy, and to assess the effect of parecoxib co-administration on the biodistribution and therapeutic efficacy of radiolabeled MN-14.

Materials and methods

Animal model of small peritoneal metastases

Male nude BALB/c mice (Charles River Laboratories, Germany), 7-9 weeks old, weighing 20-25 grams were used in the experiments. Mice were accustomed to laboratory conditions for at least one week before experimental use and were housed under nonsterile standard conditions (temperature 20–24°C; relative humidity 50–60%; 12 h light/12 h dark) in filter-topped cages (up to five mice per cage), on sawdust with free access to animal chow (Snif Voer, Soest, The Netherlands) and water. Peritoneal metastases were induced as described previously.[27] Briefly, mice were inoculated intraperitoneally with 1.0 x 10^6 LS174T cells (CCL 188, American Type Culture Collection, Rockville, MD), suspended in 500 µl of RPMI-1640 medium using a 23-gauge needle. In this model, the first macroscopic tumor nodules are seen seven to ten days thereafter, whereas bulky peritoneal carcinomatosis develops three to six weeks after tumor cell inoculation.
Before initiating the experimental studies, the COX-2 expression of LS174T was immunohistochemically confirmed using a murine anti-COX-2 polyclonal antibody (Brunschwig Chemie, Amsterdam, The Netherlands), as shown in Figure 1. The experiments were approved by the institutional Animal Welfare Committee of the University Medical Center Nijmegen and conducted in accordance with the principles set forth by the revised Dutch Act on Animal Experimentation (1997).

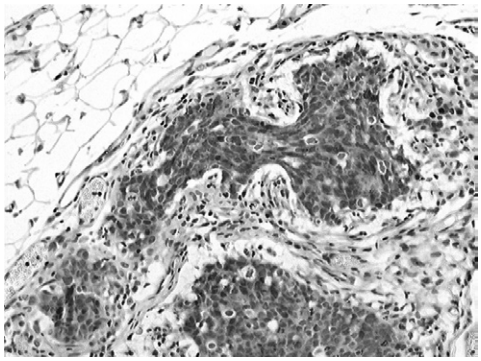

Figure 1
Anti-COX-2-immunohistochemical staining of a small, intraperitoneally growing LS174T xenograft, showing pronounced expression of the COX-2 enzyme (magnification = 200x).

Monoclonal antibody

The murine MN-14 MAb is a high-affinity (K_a = 10^9 M^{-1}) class-III anti-CEA IgG_1 antibody, produced by a hybridoma cell line culture, kindly provided by Immunomedics, Inc. (Morris Plains, New Jersey, USA).[37] The antibodies were purified by protein A chromatography, as described previously.[38] Purity was checked by SDS-PAGE under nonreduced conditions and by means of fast protein liquid chromatography (FPLC) on a Phenomenex Biosep 3000 column, eluted with phosphate buffered saline (PBS, pH 7.2, 1 mL/min).

Radioiodination and quality control

Antibodies were radioiodinated with ^{125}I (Amersham, Den Bosch, The Netherlands) or ^{131}I (MDS Nordion, Fleurus, Belgium) using the iodogen-method (1,3,4,6-tetrachloro-3α,6α-diphenyl-glycoluril; Pierce, Rockford, IL). Briefly, antibodies and ^{125}I or ^{131}I were incubated at room temperature in 85 µl of PBS (0.10 M, pH 7.4) in a glass vial, coated with 50-100 µg iodogen. After twelve minutes, the reaction mixture was separated on a PD-10 column (Amersham Biosciences, Uppsala, Sweden), eluted with PBS, 0.5% bovine serum albumin (BSA). Labeling efficiency of the radioiodination reactions exceeded 70%. In the biodistribution study the specific activity of the primary ^{125}I-MN-14 preparation was 2.9 µCi/µg. Specific activity of the primary ^{131}I-MN-14 preparation in the therapy study was 23.4 µCi/µg. In a previous study we demonstrated that the uptake of the radiolabeled MN-14 antibody in tumor was optimal at MN-14 protein doses up to 25 µg.[27] Therefore, in the present study the radiolabeled antibody preparations were augmented with unlabeled MN-14 to a total antibody protein dose of 20 µg per mouse.
The amount of free iodine was determined by instant thin layer chromatography (ITLC) with ITLC silica gel strips (Gelman Sciences, Inc., Ann Arbor, MI, USA) using

0.1 M citrate buffer (pH 6.0) as the mobile phase. Radiochemical purity of the radioiodinated antibody preparations used in the studies exceeded 98%. The immunoreactive fraction (IRF) at infinitive antigen excess of the radiolabeled MN-14 preparation was determined on freshly trypsinized LS174T cells essentially as described by Lindmo et al.[39] with minor modifications. Briefly, a fixed amount of labeled antibody (10,000 cpm) was incubated with increasing concentrations of LS174T tumor cells (1.2×10^6 – 20×10^6 cells/mL) in 0.5 mL binding buffer (RPMI medium containing 0.5% BSA and 0.05% NaN_3). A duplicate of the lowest cell concentration was incubated in the presence of an excess unlabeled antibody to correct for non-specific binding. After six hours of incubation at 37°C, the cells were washed and activity in the pellet was determined in a well-type gamma-counter. The inverse of the tumor cell bound fraction was plotted against the inverse of the cell concentration and the immunoreactive fraction (IRF) was calculated from the Y-axis intercept. The IRFs of the ^{125}I-MN-14 and ^{131}I-MN-14 preparations were 80% and 76%, respectively. The radiolabeled antibody preparations were administered within two hours after radiolabeling.

Parecoxib

Parecoxib sodium (Dynastat®), a selective COX-2 inhibitor specifically developed for parenteral administration, was purchased from Pharmacia Europe EEIG (Buckinghamshire, UK) as a powder in glass vials (40 mg/vial). Parecoxib was dissolved in 0.9% sodium chloride (saline) to the appropriate concentrations immediately before use and administered intraperitoneally (0.2 ml/mouse).

Therapeutic efficacy of parecoxib monotherapy

To evaluate the therapeutic efficacy of parecoxib when given as monotherapy, mice bearing small peritoneal metastases were treated with daily intraperitoneal administrations of increasing doses of parecoxib (0.2, 1.0, 5.0, or 25.0 mg/kg) for fourteen consecutive days, starting on the tenth day after tumor cell inoculation. Control mice received saline only (eight mice per group). Prior to each administration, body weight, as a surrogate measure of toxicity, was measured. Thirty days after tumor cell inoculation, all mice were euthanized by O_2/CO_2-asphyxiation and dissected. At dissection the intraperitoneal tumor load was score semiquantitavely, according to the peritoneal cancer index (PCI) as described by Eggermont et al.[40] In brief, the intraperitoneal tumor load could be given a PCI of 0, 1, 2, or 3, where 0 indicated no macroscopic tumor growth, 1 meant ≤ 3 pin-point tumor foci that have a diameter of ≤ 1 mm, 2 indicated moderate tumor growth, and 3 indicating abundant intraperitoneal tumor growth, replacing most of the peritoneal cavity. Subsequently, after designating a PCI rate, all macroscopic tumor deposits were meticulously excised and weighed.

Effect of parecoxib on the biodistribution of radioiodinated MN-14

To assess the effect of daily parecoxib therapy on the biodistribution of radioiodinated MN-14, ten days after tumor cell inoculation 45 mice received an i.p. injection of ^{125}I-MN-14 (5 μCi/20 μg/mouse). Parecoxib therapy, which consisted of daily intraperitoneal administrations of 1.0 mg/kg or 5.0 mg/kg per mouse, was started concurrently with the administration of the radiolabeled antibodies. Control mice received saline only. Mice were euthanized by O_2/CO_2-asphyxia and dissected at 24 hours (i.e. after one administration of parecoxib), 72 hours (i.e. after two administration of parecoxib) and 168 hours (i.e. after six administrations of parecoxib) after the administration of ^{125}I-MN-14 (five mice per group). Tumor, blood, liver, spleen, kidney, small intestine, cecum, lung and muscle tissues were sampled, gently blotted dry, and immediately weighed. Radioactivity was measured in a shielded well-type gamma-counter (Wizard, Pharmacia-LKB, Sweden). To correct for physical decay and to calculate the uptake of the radioiodinated antibody in each sample as a fraction of the injected dose, aliquots of the injected dose were counted simultaneously. The results were expressed as percentage of the injected dose per gram tissue (% ID/g).

Combination therapy of RIT and parecoxib

To assess the radiosensitizing effect of parecoxib when combined with RIT, ten days after tumor cell inoculation groups of twelve mice each were treated with either ^{131}I-MN-14 (125 μCi/mouse, which is approximately 25% of the maximal tolerated dose), parecoxib (either 1.0 mg/kg or 5.0 mg/kg/day for fourteen consecutive days) or ^{131}I-MN-14 combined with parecoxib. ^{131}I-MN-14 was given intraperitoneally on day 10 in 200 μl of PBS, 0.5% BSA. Parecoxib was given daily by intraperitoneal injections in from day 10 till 23. Control mice received daily intraperitoneal injections of 200 μl of saline. Mice treated with RIT only, were given daily injections of 200 μl of saline. Prior to each administration, the mice were weighted to ensure that body weight loss did not exceed 20%. Mice were monitored daily and body weight was measured daily in the first two weeks after the first administration. Mice were monitored until the body weight had dropped more than 20% or until the humane endpoint had been reached, as determined by an experienced and independent animal technician, who was blinded to the therapeutic regimen. At the time of the humane endpoint, mice were usually cachectic and drowsy, showing signs of advanced peritoneal carcinomatosis, such as the presence of bloody ascites or bulky intraperitoneal tumor growth, and were expected to die with one or two days. When body weight had dropped more than 20% or the humane endpoint had been reached, mice were euthanized by O_2/CO_2-asphyxiation and immediately dissected. At dissection, tumor load was scored by the peritoneal cancer index (PCI), as described above, and all tumor deposits were excised and weighed. The experiment was terminated at 118 days after tumor cell inoculation when the remaining mice were euthanized and dissected. The ab-

dominal cavity was carefully inspected. Liver, spleen, lungs, pancreas, greater omentum and the diaphragm were removed for routine histopathological H&E-staining and immunohistochemical staining using a rabbit-anti-human anti-CEA polyclonal antibody (A 0115, DakoCytomation, Glostrup, Denmark).

Statistical analysis

Statistical analysis was performed by means of the GraphPad Prism 4.00 software (GraphPad Software, San Diego, CA). Comparisons were analyzed using the one-way ANOVA test. Bonferroni correction for multiple testing was applied. Survival curves were compared using the Log-rank test. All tests were two-sided; the level of statistical significance was set a P-value of <0.05.

Results

Parecoxib monotherapy

Fourteen daily administrations of parecoxib, when given as sole treatment, had no influence on body weight at any of the dose levels tested. Thirty days after tumor cell inoculation, one mouse of the control group, had abundant intraperitoneal tumor growth. At that time it was decided to terminate the experiment and dissect all animals. At dissection, all mice of the control group that had been treated with saline had macroscopic tumor growth. Of the mice treated with 0.2 mg/kg or 1.0 mg/kg per ad-

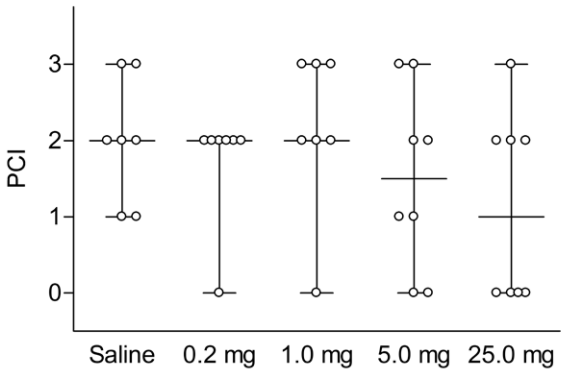

Figure 2
The peritoneal cancer index (PCI) designated to mice with small intraperitoneal LS174T xenografts after treatment with either daily intraperitoneal administrations of saline or increasing dosages of parecoxib (0.2 – 25.0 mg/kg) during fourteen consecutive days. Lines indicate the median and range. There was a borderline significant trend towards a lower PCI at higher parecoxib dosages (P=0.097).

ministration, one mouse of each group no macroscopic tumor growth. Of those mice that had been treated at dose levels of 5.0 mg/kg or 25.0 mg/kg per administration, two out of eight and four out of eight had no macroscopic tumor growth, respectively. The PCI designated to the mice is depicted in Figure 2. The median PCI (range) varied between 2 (1-3) in the control group and 1 (0-3) in the mice treated with parecoxib at 25.0 mg/kg. There was a borderline significant trend towards a lower PCI at higher parecoxib dosages (P=0.097). In Figure 3, the tumor weight found in each mouse is given. The mean (± SD) tumor weight varied between 1.3 (± 1.5) g in the control group and 0.5 (± 0.4) g in the mice treated with parecoxib at 0.2 mg/kg (P=0.15 for trend). There was a highly significant correlation between the PCI and the weight of the dissected tumor deposits (Spearman r=0.9349, P<0.0001).

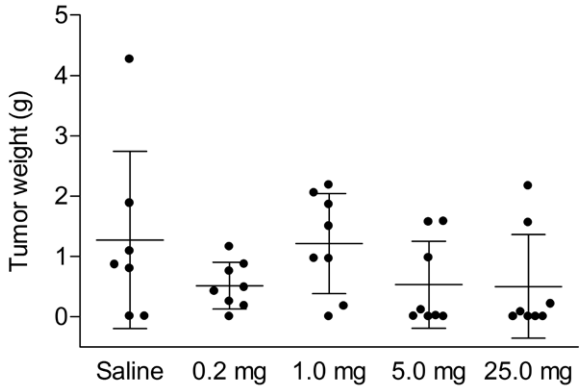

Figure 3
The tumor weight found in the mice with small intraperitoneal LS174T xenografts after treatment with either daily intraperitoneal administrations of saline or increasing dosages of parecoxib (0.2 – 25.0 mg/kg) during fourteen consecutive days. Lines indicate mean ± SD.

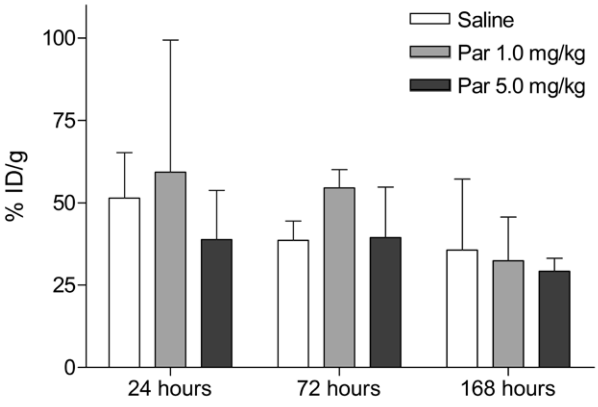

Figure 4
The uptake of ^{125}I-MN-14 in tumor expressed as percentage of the injected dose per gram (% ID/g), with of without daily intraperitoneal injections of parecoxib (1.0 mg/kg or 5.0 mg/kg). Control mice received daily administrations of saline. Par, parecoxib.

Table 1. **Biodistribution of intraperitoneally administered ^{125}I-MN-14 in mice with small intraperitoneal LS174T xenografts with or without daily intraperitoneal injections of parecoxib**

	24 hours p.i. radioantibody			72 hours p.i. radioantibody			168 hours p.i. radioantibody		
Tissue	**Controls**	**Par 1.0 mg/kg**	**Par 5.0 mg/kg**	**Controls**	**Par 1.0 mg/kg**	**Par 5.0 mg/kg**	**Controls**	**Par 1.0 mg/kg**	**Par 5.0 mg/kg**
Tumor	51.4 ± 13.8	41.8 ± 10.3	38.8 ± 15.0	38.6 ± 5.8	54.5 ± 5.6	39.4 ± 15.4	35.6 ± 21.6	32.3 ± 13.3	29.2 ± 4.0
Blood	15.3 ± 3.1	14.9 ± 4.4	13.6 ± 2.1	11.1 ± 3.2	14.4 ± 1.3	10.7 ± 2.7	7.7 ± 2.9	7.4 ± 3.1	6.9 ± 2.6
Muscle	1.9 ± 0.3	1.6 ± 0.4	1.7 ± 0.3	0.8 ± 0.5	1.5 ± 0.2	1.1 ± 0.3	0.7 ± 0.2	0.7 ± 0.2	0.7 ± 0.2
Lung	7.1 ± 2.9	10.8 ± 4.0	10.7 ± 1.9	8.2 ± 2.7	12.9 ± 2.0	10.4 ± 3.2	4.7 ± 1.8	6.8 ± 2.6	5.4 ± 2.3
Pancreas	3.5 ± 2.5	2.0 ± 0.6	2.3 ± 0.7	1.2 ± 0.3	1.6 ± 0.5	1.3 ± 0.2	0.8 ± 0.3	0.8 ± 0.4	0.8 ± 0.3
Spleen	2.7 ± 0.7	2.4 ± 0.8	2.0 ± 0.6	2.2 ± 0.4	2.7 ± 0.5	1.5 ± 1.1	1.3 ± 0.5	1.2 ± 0.6	1.0 ± 0.5
Kidney	3.8 ± 0.7	3.7 ± 1.1	3.6 ± 0.7	2.8 ± 0.7	4.1 ± 0.7	3.0 ± 0.8	2.0 ± 0.7	2.1 ± 0.9	1.8 ± 0.6
Liver	5.2 ± 0.9	4.0 ± 1.2	3.9 ± 0.9	3.0 ± 0.6	2.8 ± 1.9	2.8 ± 0.9	1.9 ± 0.7	1.9 ± 0.9	1.9 ± 0.8
Small intestine	2.7 ± 0.5	3.0 ± 1.0	2.7 ± 0.6	2.3 ± 0.8	3.3 ± 0.6	2.2 ± 0.8	1.2 ± 0.5	1.4 ± 0.6	1.0 ± 0.3
Cecum	4.2 ± 1.1	4.9 ± 1.4	4.4 ± 1.0	3.5 ± 1.4	4.8 ± 0.7	3.4 ± 1.2	2.4 ± 1.1	2.6 ± 1.4	2.2 ± 1.1
Tumor-to-blood ratio	3.4 ± 0.4	2.4 ± 1.4	2.8 ± 0.9	3.7 ± 1.2	3.6 ± 0.6	3.6 ± 0.9	5.0 ± 1.5	4.5 ± 0.7	4.7 ± 1.7

Par, parecoxib. Values are given as means ± SD (five mice per group)

Influence of parecoxib on the biodistribution of radioiodinated MN-14

The results of the experiment in which the effect of parecoxib on tumor uptake and biodistribution of ^{125}I-labeled MN-14 was studied, are summarized in Table 1. Parecoxib co-administration had no effect on the uptake of ^{125}I-MN-14 in tumor or on the blood levels or tumor-to-blood ratios. Mean uptake of ^{125}I-MN-14 in tumor nodules obtained from the control mice at 24, 72 and 168 hours post-injection (p.i.) amounted 51.4 (± 13.8)% ID/g, 38.6 (± 5.8)% ID/g and 35.6 (± 21.6)% ID/g, respectively. Tumor uptake of ^{125}I-MN-14 in the mice that received daily administration of either 1.0 mg/kg or 5.0 mg/kg parecoxib was very similar to that in control mice, as shown in Figure 4. Furthermore, parecoxib co-administration had no influence on the normal tissue distribution of ^{125}I-MN-14 at the various time points tested.

Combination therapy of parecoxib and ^{131}I-labeled MN-14

To test the hypothesis that the COX-2 inhibitor parecoxib might improve the efficacy of RIT in small peritoneal metastases, parecoxib was administered daily for fourteen consecutive days, starting concurrently with the administration of ^{131}I-MN-14.
Control mice that were treated with daily injections of saline showed a maximum weight loss of 1.9 ± 3.9% at four days after the start of treatment. RIT alone using 125 μCi ^{131}I-MN-14 per mouse resulted in a maximum weight loss of 3.9 ± 1.5% four days after administration of the radiolabeled antibodies. The toxicity of RIT was not affected by co-administration of parecoxib. Maximum weight loss after RIT combined

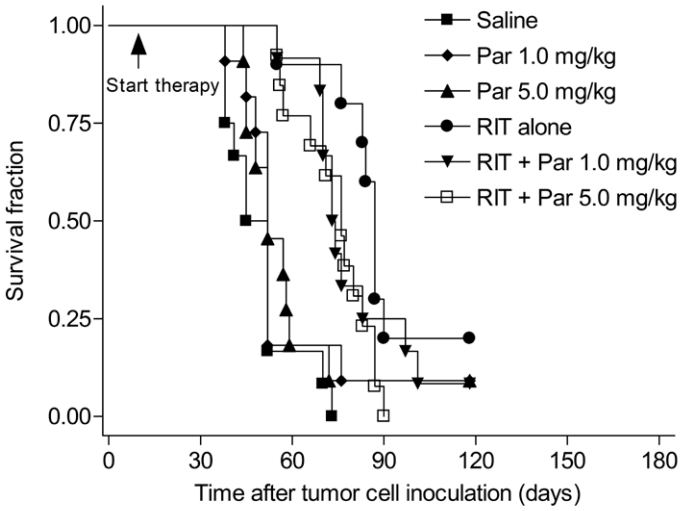

Figure 5
Kaplan-Meier survival plot of mice with small intraperitoneal LS174T xenografts after intraperitoneal administration of ^{131}I-MN-14 (125 μg/mouse), parecoxib monotherapy (fourteen daily administrations at 1.0 mg/kg or 5.0 mg/kg), or RIT combined with parecoxib. Par, parecoxib.

with daily administrations of parecoxib at 1.0 mg/kg or 5.0 mg/kg was 3.5 ± 2.6% and 4.8 ± 0.9, respectively (P=0.42). There were no treatment-related deaths.
Five mice (7%) developed large subcutaneous tumors at the inoculation site and were excluded from the survival analysis. The survival curves for the various treatment groups are shown in Figure 5. Median survival of the control mice was 48.5 days (range 38-73). Parecoxib monotherapy at 1.0 mg/kg or 5.0 mg/kg resulted in a median survival of 52 days (range 38-118) and 52 days (range 44-118), respectively (P=0.47). RIT alone resulted in a significantly improved median survival of 87 days (range 55-118, P<0.0001). Daily intraperitoneal injections of parecoxib at 1.0 mg/kg or 5.0 mg/kg during fourteen consecutive days after the administration of ^{131}I-MN-14 resulted in a median survival of 73.5 (range 55-118) and 76 (range 55-90) days, respectively (P=0.15, comparing RIT alone with RIT + parecoxib 1.0 mg/kg or 5.0 mg/kg). Although the difference between the survival of the mice treated with RIT alone and the mice treated with RIT + parecoxib 1.0 mg/kg was not significant (P=0.19), median survival of the mice treated with RIT alone was significantly better than that of the mice treated with RIT + parecoxib 5.0 mg/kg (P=0.029).
At dissection, the PCI was 3 for all control mice. Median PCI for the mice treated with parecoxib 1.0 mg/kg, parecoxib 5.0 mg/kg, RIT alone, RIT + parecoxib 1.0 mg/kg and RIT + parecoxib 5.0 mg/kg was 2.6 (range 2-3), 3 (range 2-3), 3 (range 2-3), 3 (range 2-3) and 3 (range 2-3), respectively (P=0.56). Figure 6 depicts the tumor weight that was found at dissection at the time mice reached their humane endpoints. It varied between 4.3 ± 1.4 for the mice treated with parecoxib 5.0 mg/kg and 4.9 ± 1.6 for the mice treated with RIT + parecoxib 5.0 mg/kg (P=0.94).

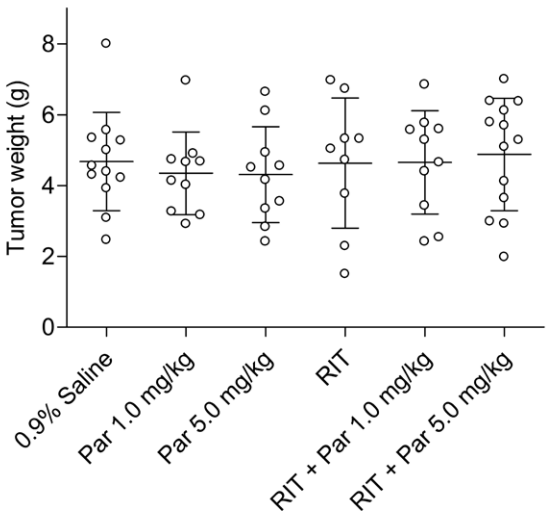

Figure 6
Scatter dot plot, specifying the tumor weight found at dissection. Lines indicate means ± standard deviation. The variation between the groups is not statistically different (P=0.94). ***Par, parecoxib.***

The highly similar tumor mass found in the mice demonstrates that there was no bias in determining the humane endpoint. The method of determining and comparing the survival was therefore reliable.
At the end of the experiment (118 days after tumor cell inoculation), there were five long-term survivors (two treated with RIT alone; one treated with RIT + parecoxib 1.0 mg/kg; and two treated with parecoxib monotherapy at 1.0 mg/kg and 5.0 mg/kg, respectively), without signs of intraperitoneal tumor growth. At dissection, the mouse that had been treated with parecoxib monotherapy at 5.0 mg/kg had macroscopic tumor growth (0.36 g; PCI 2), whereas in the remaining four mice there was no evidence of disease. Histopathological examination of relevant organs, including the greater omentum, mesentery, diaphragm and pancreas did not reveal residual disease in any of these mice.

Discussion

The primary aim of the current study was to investigate the potential of the COX-2 inhibitor parecoxib to act as a radiosensitizer when combined with RIT in a well-characterized animal model of small volume peritoneal carcinomatosis of colonic origin. Daily administration of parecoxib, however, failed to improve the efficacy of RIT using ^{131}I-MN-14 in this model.

The COX-2 inhibitor parecoxib was selected, since it has several favorable properties that made it attractive as a potential radiosensitizer. In contrast to other commercially available selective COX-2 inhibitors that are poorly soluble in water and can only be given orally, parecoxib is water-soluble and was specifically developed for parenteral (intramuscular or intravenous) administration. As such, parecoxib allows more precise dosing. After hydrolysis in the liver, the active metabolite valdecoxib binds non-covalently to COX-2, forming a firm and stable enzyme-inhibitor complex.[41] Valdecoxib has been shown to have a higher ***in vitro*** affinity to COX-2, and bind more rapidly to and dissociate from COX-2 more slowly than celecoxib,[42,43] which has been one of the most used and studied COX-2 inhibitors. Although there are no published data on the pharmacokinetics of parecoxib in mice, experimental studies in rats have shown that after intravenous administration parecoxib is rapidly converted to valdecoxib (conversion time, 8 minutes), which then has a plasma half-life of four to five hours.[34] Zhang et al. studied the pharmacokinetics of [^{14}C]Valdecoxib after oral ingestion at 5 mg/kg in mice, and found a peak plasma concentration at 30 minutes after ingestion and the half-life in blood of approximately four hours, with most of the [^{14}C]Valdecoxib associated with the red blood cells.[44]
COX-2 inhibition with parecoxib has been shown to have antitumor effects in experimental breast cancer and colon cancer.[35,36] O'Donoghue et al.[35] investigated the therapeutic efficacy of fifteen daily intraperitoneal administrations of parecoxib (0.5 mg/kg) in mice bearing subcutaneous breast cancer xenografts and observed growth

inhibition of the primary tumor and pulmonary metastases. Smakman et al. studied the efficacy of twice-daily intraperitoneal injections of parecoxib (5 mg/kg) during six days in mice immediately after inducing liver metastases of colon carcinoma and reported greatly reduced intrahepatic tumor cell proliferation and the rate of liver metastases outgrowth.[36] Therefore, before combining parecoxib with RIT in the current model of peritoneal carcinomatosis, we first investigated the toxicity and efficacy of parecoxib monotherapy. Parecoxib proved to be a safe agent; body weight loss among the treatment groups was similar to that of the control group, and none of the mice had gastric ulcers at dissection. Daily intraperitoneal injections of parecoxib during fourteen consecutive days had no statistically significant antitumor effect in this study, although there was a borderline trend towards a dose-dependent antitumor effect. At the highest dose level (25.0 mg/kg) four out of eight mice had no macroscopic tumor growth, which suggests that high doses of parecoxib might have an antitumor effect in this model.

COX-2 derived prostaglandins play an important role in tumor angiogenesis.[45] Selective inhibition of COX-2 has been shown to be associated with a decrease in new vessel formation.[46,47] Therefore, it could not be excluded that COX-2 inhibition with parecoxib might affect the tumor uptake of radiolabeled antibodies in RIT, which prompted us to conduct the biodistribution study. Daily administration of parecoxib at 1.0 mg/kg or 5.0 mg/kg had no effect on the overall biodistribution and tumor uptake in particular, of radioiodinated MN-14 in this model.

To date, several papers have been published reporting synergistic or radiosensitizing activity of COX-2 inhibition when given prior to and concurrently with external beam gamma-irradiation in various epithelial cancers.[18] Celecoxib (Celebrex®) or its bioactive compound SC-236, has been the COX-2 inhibitor used in most studies. In view of the pronounced expression of COX-2 in LS174T, and because the efficacy of RIT at relatively low activity doses is limited, we hypothesized that inhibition of the COX-2 enzyme might have a synergistic or radiosensitizing effect when combined with RIT in this model. RIT was given at 25% of the MTD, since we previously demonstrated that this activity dose significantly delays the development of peritoneal carcinomatosis but is not curative in this model. Thus, we assumed that a clinically relevant radiosensitizing effect of parecoxib could be demonstrated at this dose level. Since the radiosensitizing effect of celecoxib has been demonstrated to be dose-dependent in external beam radiation,[20] parecoxib was combined with RIT at two dose levels in the current study. In concert with the results of the first experiment, in which parecoxib had no significant antitumor effect, the median survival of the mice treated with parecoxib monotherapy was similar to that of the control mice. RIT alone significantly improved the median survival as compared to that obtained in the control mice. Daily intraperitoneal administrations of parecoxib, however, did not enhance the efficacy of RIT, and, of unknown significance, appeared even to be detrimental to the effect of RIT at the higher dose level of parecoxib (5.0 mg/kg). All mice were euthanized at the time their clinical condition had deteriorated beyond the humane endpoint due

to abundant intraperitoneal tumor growth. Mean tumor weight of all the treatment groups was very similar. Therefore, long-term adverse systemic effects of the COX-2 inhibition seem to be an unlikely explanation for the observed survival difference between the mice treated with RIT alone and those treated with RIT + parecoxib 5 mg/kg.

Special features of the published experimental models, in which COX-2 inhibitors were successfully applied as radiosensitizers, that might explain the failure of parecoxib to act as a radiosensitizer in the current model, include the type of radiotherapy (external beam radiation versus RIT), the animal model with respect to the localization of the xenografts (subcutaneous versus intraperitoneal), and the choice of the COX-2 inhibitor. RIT differs from external beam radiation in that in RIT the radiation energy is deposited over a long time interval (low dose rate), whereas in external beam radiation, the radiation energy is delivered in one or more fractions at a high dose rate.[48] Inhibition of the COX-2 enzyme might only sensitize tumor cells to radiation when given at a high dose rate. Furthermore, because of the long time-interval during which the radiation energy is delivered to the tumor nodules in RIT, it seems unlikely that a radiosensitizing effect of parecoxib would have become apparent had the drug been administered prior to the injection of the radiolabeled antibodies.

Another explanation of the absence of any radiosensitizing activity by parecoxib may be the intraperitoneal localization of the tumor xenografts, as opposed to the subcutaneous xenografts, used in other studies reporting a radiosensitizing effect when COX-2 inhibition was combined with radiotherapy. Whereas in subcutaneous tumor models therapeutic efficacy is determined by measuring the growth of the subcutaneous tumors, in the current model of peritoneal carcinomatosis survival has been the primary endpoint. Failure of the animal model to reveal small differences in therapeutic efficacy might have obscured any additional therapeutic effect of parecoxib coadministration. Furthermore, although RIT alone was noncurative in eight out of ten mice, its efficacy could have outshined a potential radiosensitizing effect of parecoxib in this model.

Finally, since parecoxib is a prodrug that needs to be hydrolyzed by the liver, its active metabolite Valdecoxib can only reach the intraperitoneal tumors via the blood. It cannot be excluded that the vascularization of the small intraperitoneal LS174T xenografts is too limited to allow sufficient supply of the COX-2 inhibitor Valdecoxib. Whether intraperitoneal administration of other COX-2 inhibitors that do not need conversion could reach the intraperitoneal xenografts more effectively and thus enhance RIT of peritoneal carcinomatosis of colonic origin remains yet to be ascertained.

In conclusion, monotherapy using the COX-2 inhibitor parecoxib had no antitumor effect in nude mice with small intraperitoneal LS174T colon cancer xenografts. When given concurrently with RIT, parecoxib had no effect on the biodistribution of the radiolabeled antibodies, did not enhance the therapeutic efficacy of RIT and appeared even to have a detrimental effect on the efficacy of RIT at the highest dose tested.

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6

Combination therapy using gemcitabine and radioimmunotherapy in nude mice with small peritoneal metastases of colonic origin

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Despite the promising results of radioimmunotherapy (RIT) using radiolabeled monoclonal antibodies (MAbs) for the treatment of non-Hodgkin lymphoma (NHL), the therapeutic efficacy of RIT in solid tumors has been modest at best. Solid cancers are targeted less efficiently with radiolabeled antibodies than hematological malignancies, which has been attributed to the presence of various physiological barriers between the circulation and the tumor cell surface. It has been pointed out that the vascular endothelium, the relatively large transport distances in the tissue and the enhanced interstitial pressure in the tumor tissue, hamper the penetration of antibodies into the tumor tissue to bind to their target antigen.[1] As a result, tumor uptake of radiolabeled MAbs and, consequently, the radiation doses delivered to solid lesions are in most cases too low to induce objective tumor responses. Several innovative approaches have been pursued with the aim to improve the efficacy of RIT. These include strategies to improve the localization and retention of radiolabeled MAbs in the tumor (e.g. using high-affinity MAbs[2]), strategies to accelerate the blood clearance of the radiolabel (e.g. using antibody fragments[3] or the pretargeting method[4]), and strategies to increase the sensitivity of the tumor cells to radiation (e.g. using radiosensitizers[5-7]).

Gemcitabine (2'-deoxy-2'2'-difluorocytidinemonohydrochloride) is a pyrimidine analogue and has been shown to exert antitumor effects in a wide range of solid tumors, as well as some hematological malignancies.[8] Gemcitabine acts by depleting the deoxynucleoside triphosphate pool and is incorporated in the DNA, in the same way as 5-fluorouracil (5-FU), thereby inhibiting DNA synthesis and, probably, DNA repair.[9] Gemcitabine has been shown to sensitize a wide range of mainly epithelial cancers to radiation, including adenocarcinoma of the pancreas and colon, and squamous cell carcinoma.[10] Although in most chemoradiotherapy studies gemcitabine was combined with external beam radiation, in recent years a few studies have been published reporting radiosensitizing effects of gemcitabine when combined with RIT in animal models of pancreatic cancer and colon cancer.[5-7,11]

We have previously characterized a nude mouse model for RIT of small volume peritoneal carcinomatosis of colorectal origin using the human colon carcinoma cell line LS174T.[12] RIT using the radiolabeled anti-CEA MAb MN-14 proved very effective in inhibiting the growth of intraperitoneally growing tumor nodules, even at relatively low activity doses. ^{131}I and ^{177}Lu proved to be the most effective radionuclides in this model.[13] Since gemcitabine has previously been shown to exert some antitumor activity against LS174T[14] and improve the efficacy of RIT in a subcutaneous tumor model using LS174T,[11] we hypothesized that gemcitabine co-administration might sensitize the intraperitoneally growing LS174T xenografts to radiation and improve the therapeutic efficacy of RIT in our model of small volume peritoneal carcinomatosis of colorectal origin. To test this hypothesis, first the maximum tolerated dose (MTD) of gemcitabine was determined in two different administration regimens. Subsequently, the effect of both administration regimens of gemcitabine on the efficacy of RIT using ^{131}I-labeled MN-14 was assessed.

Materials and methods

Animal model of small peritoneal metastases

Male nude BALB/c mice (Charles River Laboratories, Germany), 8-10 weeks old, weighing 20-25 grams were used in the experiments. Mice were accustomed to laboratory conditions for at least one week before experimental use and were housed under nonsterile standard conditions (temperature 20–24°C; relative humidity 50–60%; 12 h light/12 h dark) in filter-topped cages (up to five mice per cage), on sawdust with free access to animal chow (Snif Voer, Soest, The Netherlands) and water. At the start of therapy, all mice were housed on iron grate floors. Peritoneal metastases were induced as described previously.[12] In brief, mice were inoculated intraperitoneally with 1.0 x 10^6 LS174T cells (CCL 188, American Type Culture Collection, Rockville, MD), suspended in 500 µl of RPMI-1640 medium in a 2.5 mL syringe using a 23-gauge needle. In this model, the first macroscopic tumor nodules are seen seven to ten days later, whereas bulky peritoneal carcinomatosis develops three to five weeks after tumor cell inoculation. All experiments were conducted in accordance with the principles laid out by the revised Dutch Act on Animal Experimentation (1997) and approved by the institutional Animal Welfare Committee of the Radboud University Nijmegen.

Monoclonal antibody

The murine MN-14 MAb is a high-affinity (K_a = 10^9 M^{-1}) class-III anti-CEA IgG_1 antibody, produced by a hybridoma cell line culture, kindly provided by Immunomedics, Inc. (Morris Plains, New Jersey, USA). The antibodies were purified by protein-A chromatography, as described previously.[15] Purity was checked by fast protein liquid chromatography (FPLC) on a Phenomenex Biosep 3000 column (size 300x7.8mm), eluted with phosphate buffered saline (PBS, pH 7.2, 1 mL/min).

Radioiodination

Antibodies were radioiodinated with ^{131}I (MDS Nordion, Fleurus, Belgium, respectively) using the iodogen-method (1,3,4,6-tetrachloro-3α,6α-diphenyl-glycoluril; Pierce, Rockford, IL). Briefly, antibodies and ^{131}I were incubated at room temperature in 85 µl of phosphate buffered saline (PBS 0.10 M, pH 7.4) in a glass vial, coated with 100 µg iodogen. The reaction mixture was subsequently separated on a PD-10 column (Amersham Biosciences, Uppsala, Sweden), eluted with PBS, 0.5% bovine serum albumin (BSA). Labeling efficiency of the radioiodination reactions was approximately 70%. Specific activities of the ^{131}I-MN-14 preparations were 1.6 MBq/µg (43 µCi/µg) and 0.64 MBq/µg (17 µCi/µg), respectively. In a previous study we demonstrated that the uptake of the radiolabeled MN-14 antibody in tumor was optimal at MN-14 pro-

tein doses up to 25 μg.[12] Therefore, in the present study unlabeled MN-14 was added to the radiolabeled antibody preparations to adjust the total antibody protein dose to 20 μg per mouse.

Quality control of the radiolabeled antibody preparations

The amount of free ^{131}I was determined by instant thin layer chromatography (ITLC) using ITLC silica gel strips (Gelman Sciences, Inc., Ann Arbor, MI, USA) using 0.1 M citrate buffer, pH 6.0, as the mobile phase. Radiochemical purity of all radiolabeled antibody preparations used in the studies exceeded 97%. The immunoreactive fraction (IRF) of the radiolabeled MN-14 preparations was determined on freshly trypsinized LS174T cells essentially as described by Lindmo et al.[16] with minor modifications. Briefly, a fixed amount of labeled antibody (10,000 cpm) was incubated with increasing concentrations of LS174T tumor cells (1.2×10^6 – 20×10^6 cells/mL) in 0.5 mL binding buffer (RPMI medium containing 0.5% BSA and 0.05% NaN_3). A duplicate of the lowest cell concentration was incubated in the presence of an excess unlabeled antibody to correct for non-specific binding. After six hours of incubation at 37 °C, the cells were washed and activity in the pellet was determined in a well-type gamma-counter. The inverse of the tumor cell bound fraction was plotted against the inverse of the cell concentration and the immunoreactive fraction (IRF) was calculated from the Y-axis intercept. The IRF of the ^{131}I-MN-14 preparations was 82% and 77%, respectively. The radiolabeled antibody preparations were administered within two hours after radiolabeling.

Gemcitabine

Gemcitabine (Gemzar®) was purchased from Ely Lilly Company (Houten, The Netherlands) as a powder in a glass vial (200 mg/vial). Immediately before administration, gemcitabine was dissolved in 0.9% sodium chloride (saline) to the appropriate concentrations.

Studies to determine the maximum tolerated gemcitabine dose

To determine the MTD of gemcitabine, groups of 6-8 mice were injected intraperitoneally with escalating doses of gemcitabine. Two dosing regimens were investigated in two separate experiments. In the first regimen, mice were treated on day 0, 3, 6, and 9 with gemcitabine at 0.11 mg, 0.33 mg, 1.0 mg, or 3.0 mg/mouse/administration. The second dosing regimen consisted of daily intraperitoneal administrations of 0.022, 0.066, 0.20, or 0.60 mg/mouse/administration, which was given daily on five consecutive days. Control mice received saline only. Mice were monitored daily, and body weight was measured daily to ensure that body weight loss did not exceed 20%.

Combination therapy with radioimmunotherapy and gemcitabine

To assess the radiosensitizing effect of gemcitabine, two separate therapy experiments were carried out in which each of the two gemcitabine dosing regimens described above was combined with RIT. In both experiments, RIT consisted of 125 μCi ^{131}I-MN-14/mouse, which was given intraperitoneally ten days after tumor cell inoculation (20 μg/mouse). This activity dose represents approximately 25% of the maximum tolerated activity dose of ^{131}I-labeled IgG antibodies, which has previously been shown to significantly delay the growth of intraperitoneally growing LS174T xenografts in the present model.[13] The majority of mice still develop peritoneal carcinomatosis only after 9-10 weeks. Thus, we assumed that a relevant radiosensitizing effect of gemcitabine could be demonstrated at this dose level.
In the first therapy study, mice were treated with either RIT alone (125 μCi ^{131}I-MN-14/mouse, 10 days after tumor cell inoculation), gemcitabine alone (either 0.11 mg or 0.33 mg/mouse/administration on day 10, 13, 16, and 19 after tumor cell inoculation) or ^{131}I-MN-14 combined with gemcitabine. In the second therapy study, mice were treated with either RIT alone (125 μCi ^{131}I-MN-14/mouse, 10 days after tumor cell inoculation), gemcitabine alone (0.022 mg/per/mouse on day 10, 11, 12, 13, and 14 after tumor cell inoculation), or RIT combined with gemcitabine. In both studies, control mice received unlabeled MN-14 in PBS, 0.5% BSA. Furthermore, control mice and the mice that were treated with RIT alone received intraperitoneal injections of saline instead of gemcitabine (ten to twelve mice per group).
Mice were monitored until the humane endpoint had been reached, as determined by an experienced and independent animal technician, who was blinded to which treatment mice had received.[17] At the time of the humane endpoint, mice were usually cachectic and lethargic, showing signs of advanced peritoneal carcinomatosis, such as the presence of bloody ascites or bulky intraperitoneal tumor growth, and were expected to die within one or two days. When the humane endpoint had been reached, mice were euthanized by O_2/CO_2-asphyxiation and immediately dissected. At dissection, all macroscopic tumor deposits were meticulously excised and weighed. The experiments were terminated at 134 and 96 days after tumor cell inoculation when the remaining mice were euthanized and dissected. The abdominal cavity was conscientiously inspected. Liver, spleen, lungs, pancreas, greater omentum and the diaphragm were removed for routine histopathological H&E-staining and immunohistochemical staining using a rabbit-anti-human anti-CEA polyclonal antibody (A 0115, DakoCytomation, Glostrup, Denmark).[13]

Statistical analysis

Data are expressed as means ± standard deviation (SD) unless stated otherwise. Statistical analysis was performed by means of the GraphPad Prism 4.00 software (GraphPad Software, San Diego, CA). Single comparisons were analyzed the non-

parametric Mann Whitney *U* test. Multiple comparisons were analyzed using the one-way ANOVA test. Bonferroni correction for multiple testing was applied. In the therapy studies, comparisons between the groups for differences in survival were analyzed using the Log-rank test. All tests were two-sided; the level of statistical significance was set at a *P* value of <0.05.

Results

Toxicity of gemcitabine

To determine the dose of gemcitabine that could be administered safely, mice received intraperitoneal injections of gemcitabine in two dosing regimens. In the first experiment, in which four injections of gemcitabine were given every third day, four out of six and one out of six mice that were treated at 3.0 and 1.0 mg/mouse/administration, respectively, died due to severe gemcitabine-related toxicity during the treatment period. Body weight loss exceeded 20% and mice suffered from gastrointestinal toxicity, which consisted of diarrhea, and, in some mice, hematologic toxicity, manifesting by the presence of skin petechiae. When administered at 0.33 or 0.11 mg/administration, none of the mice developed diarrhea or skin petechiae. Figure 1a depicts the relative body weight during the study. Whereas control mice showed a gradual increase in body weight, maximum average body weight loss was 2.4 ± 1.4% in the mice treated at 0.33 mg/administration (two days after the first administration) and 1.7 ± 2.2% in the mice treated at 0.11 mg/administration (one day after second administration; P=0.24).

In the second experiment, in which five daily injections of gemcitabine were given, all mice treated at 0.60 mg/administration or 0.20 mg/administration and six out of eight mice treated at 0.066 mg/administration died due to severe gemcitabine-related toxicity either during or shortly after the treatment period. Daily administration at 0.022 mg/administration did not result in clinically evident toxicity. As shown in Figure 1b, the maximum average body weight loss amounted 6.4 ± 3.6% seven days after the first administration, which did not differ significantly from the maximum weight loss of 4.8% ± 3.6% in the control mice given daily injections of saline (P=0.19).

Based on these observations, gemcitabine was considered safe when administered at 0.33 mg/mouse/administration every third day for a total of four administrations, or at 0.022 mg/mouse/administration, when administered daily for five consecutive days.

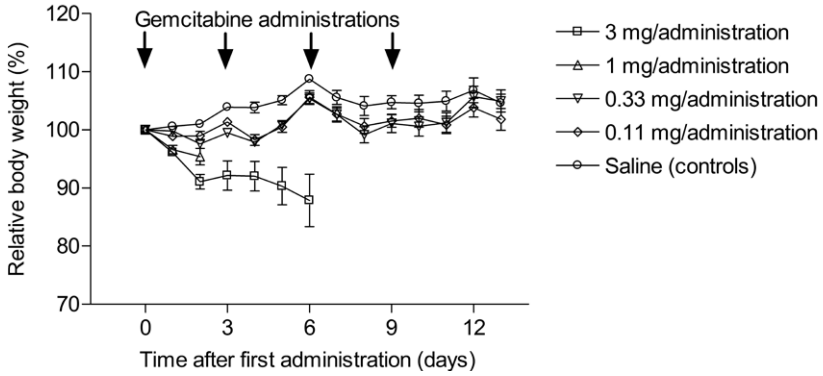

Figure 1a

Relative body weight of nude mice after once-daily intraperitoneal administration of gemcitabine at 0, 0.11, 0.33, 1.0 or 3.0 mg/administration every third day for a total of four administrations. Data respresent means ± standard error of the mean (SEM).

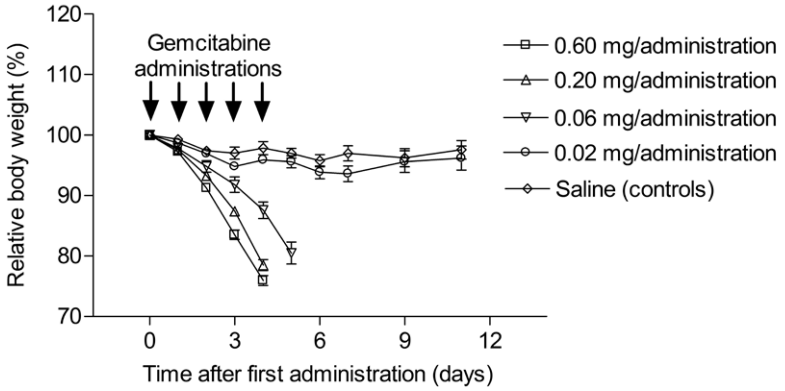

Figure 1b

Relative body weight of nude mice after once-daily intraperitoneal administration of gemcitabine at 0, 0.022, 0.066, 0.20 or 0.60 mg/administration for a total of five administrations. Data represent means ± SEM.

RIT combined with gemcitabine administered every third day for four consecutive days

The survival curves for the various treatment groups are shown in Figure 2A. Median survival of the control mice was 39 days (range 34-52). Gemcitabine monotherapy at 0.11 mg or 0.33 mg/mouse/administration every third day for a total of four administrations resulted in a median survival of 52 days (range 48-62) and 57 days (range 52-69), respectively (P=0.0003 for trend when compared to controls). RIT alone resulted in a significantly improved median survival of 66 days (range 55-143, P<0.0001 compared to controls). RIT combined with four administrations of gemcitabine at 0.11 mg or 0.33 mg/mouse/administration every third day resulted in a median survival of 73 days (range 66-101) and 94 days (range 66-143), respectively (P=0.12 for trend compared to RIT alone).
The tumor weight found at dissection at the time mice reached their humane endpoints varied between 4.0 ± 1.4 g for the mice treated with gemcitabine monotherapy 0.11 mg/administration and 5.4 ± 1.1 g for the mice treated with RIT + gemcitabine 0.33 mg/administration (P=0.24). The similar tumor mass found in the mice in the various treatment groups demonstrates that there was no bias in determining the humane endpoint. The method of determining and comparing the survival was therefore reliable.
At the end of the experiment (143 days after tumor cell inoculation), there were four long-term survivors (two treated with RIT alone; and two treated with RIT + gemcitabine 0.33 mg/mouse/administration), without signs of intraperitoneal tumor growth. At dissection, two mice (one from each group) had macroscopic tumor growth (0.13 g and 0.06 g, respectively), whereas in the remaining two mice there was no evidence of disease. Histopathological examination of relevant organs, including the greater omentum, mesentery, diaphragm and pancreas did not reveal residual disease in any of these mice.

RIT combined with gemcitabine administered daily for five consecutive days

The survival curves for the various treatment groups are shown in Figure 2B. Median survival of the control mice was 48 days (range 31-58). Daily gemcitabine monotherapy at 0.022 mg/mouse/administration for five consecutive days resulted in a median survival of 49 days (range 34-73, P=0.17 compared with controls). RIT alone resulted in a significantly improved median survival of 66 days (range 45-96, P=0.0010 compared with controls). Five daily gemcitabine administrations at 0.022 mg/mouse/administration starting concurrently with RIT resulted in a median survival of 65 days (range 49-91), which did not differ significantly from the survival of the mice treated with RIT alone (P=0.43).

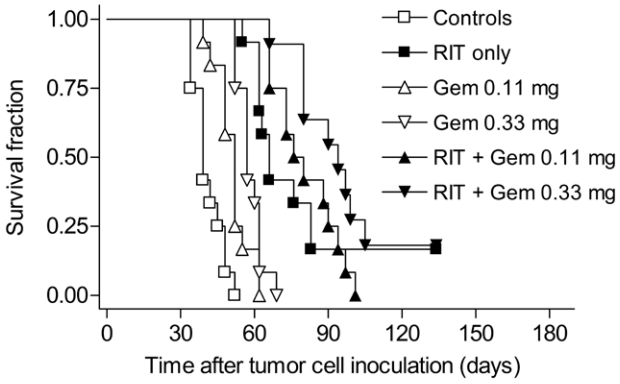

Figure 2a

Survival curves of mice with peritoneal LS174T tumor xenografts after intraperitoneal treatment with either gemcitabine alone (either 0.11 mg or 0.33 mg/mouse/administration on day 10, 13, 16, and 19 after tumor cell inoculation), RIT alone, (^{131}I-MN-14, 125 μCi/mouse on day 10 after tumor cell inoculation), or RIT combined with gemcitabine. Control mice received unlabeled MN-14 (10-12 mice per group).

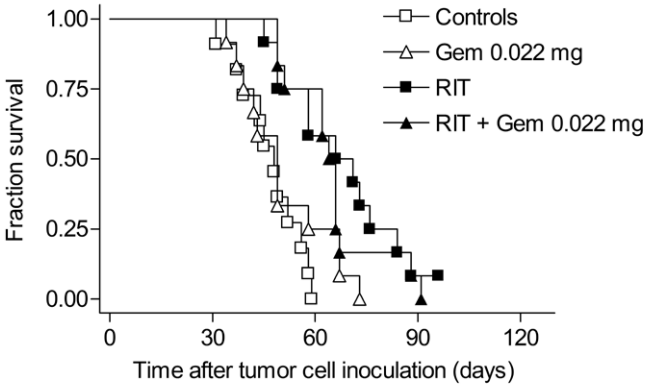

Figure 2b

Survival curves of mice with peritoneal LS174T tumor xenografts after intraperitoneal treatment with either gemcitabine alone (0.022 mg/mouse/administration on day 10, 11, 12, 13, and 14 after tumor cell inoculation), RIT alone, (^{131}I-MN-14, 125 μCi/mouse on day 10 after tumor cell inoculation), or RIT combined with gemcitabine. Control mice received unlabeled MN-14 (10-12 mice per group).

The tumor weight that was found at dissection at the time mice reached their humane endpoints varied from 4.4 ± 1.9 g for the mice treated with RIT + gemcitabine and 6.0 ± 2.0 g for the mice treated with gemcitabine monotherapy (P=0.15).
At the end of the experiment (96 days after tumor cell inoculation), one mouse treated with RIT only was still alive, without signs of intraperitoneal tumor growth. At dissection, this mouse had no macroscopic tumor growth. Histopathological examination of relevant organs, including the greater omentum, mesentery, diaphragm and pancreas did not reveal residual disease.

Discussion

Radiosensitization can be defined as the use of agents with the aim to increase the sensitivity of tissue to radiation therapy. The primary aim of the present study was to investigate the radiosensitizing potential of two gemcitabine treatment schedules when combined with single-dose radioimmunotherapy in a well-characterized animal model of peritoneal carcinomatosis of colorectal origin. Gemcitabine, however, failed to significantly improve the efficacy of radioimmunotherapy in both administration regimens tested.
Originally intended for development as an antiviral agent, gemcitabine was introduced in the late nineteen eighties as a novel and very potent pyrimidine antimetabolite, with antitumor activity against a wide range of epithelial cancers, including non-small cell and small cell lung cancer, breast cancer, pancreatic cancer, head-and-neck squamous cell cancer, and cervical cancer.[18] Furthermore, probably because of its interference with DNA repair, gemcitabine has shown to be a potent radiosensitizer. Although only marginally effective when administered as a single agent in patients with advanced colorectal carcinoma,[19] gemcitabine has been demonstrated to sensitize colorectal carcinoma to radiation therapy in both preclinical and clinical studies.[20,21] Interestingly, gemcitabine can induce radiosensitization at tissue concentrations 1,000 times lower than typical plasma levels obtained with the drug.[21] Furthermore, the radiosensitizing effects of gemcitabine have been shown to be dose-dependent.[18] This prompted us to conduct the experimental studies described in the present paper.
To date, four reports have been published on the combination of experimental RIT and gemcitabine, three of which are in animal models of pancreatic cancer,[5-7] and one in an animal model of colon cancer.[11] The group of Goldenberg published three reports on the efficacy of combination therapy using ^{131}I-labeled or ^{90}Y-DOTA-labeled chimeric PAM4 MAb and gemcitabine in nude mice bearing subcutaneous CaPan1 human pancreas carcinoma xenografts. In their first study, mice bearing subcutaneous tumors of about 1 cm^3 were treated with ^{131}I-cPAM4 (100 or 200 μCi) alone, gemcitabine alone on day 0, 3, 6, 9, and 12 at 333 mg/m^2 (2 mg/mouse), or the combination of both treatments.[5] Whereas both monotherapies did not have antitumor effects, the

combination of both treatment modalities showed a statistically significant synergistic effect, which led the authors to conclude that gemcitabine lowered the threshold for antitumor response sufficiently, so that the radiation dose provided by the low-dose RIT could still carry antitumor activity. Furthermore, combination therapy was well tolerated, even at the higher RIT activity dose of 200 μCi. In their second study, mice with somewhat smaller subcutaneous tumors (0.5 cm^3) were treated with either three cycles of 25 μCi ^{90}Y-DOTA-labeled cPAM4 only at week 0, 4 and 7, or once weekly intraperitoneal injections of gemcitabine (6 mg/mouse, which equals 1000 mg/m^2/week), or combination therapy.[7] Both RIT and gemcitabine monotherapy when given as monotherapy had some antitumor effects. When combined, however, there was a supra-additive antitumor effect, at the cost of only minimal toxicity to normal tissues, as evidenced by an average loss in body weight of less than 2% for all treatment groups. In their last report, RIT using ^{90}Y-DOTA-labeled cPAM4 was combined with gemcitabine according to the same dose regimen as utilized in their first study with ^{131}I-cPAM4 in mice bearing 1 cm^3 tumors.[6] Gemcitabine was not effective when given as single treatment, but still improved the efficacy of RIT when given in combination with ^{90}Y-DOTA-cPAM4. Finally, Graves et al. reported on the radiosensitizing effect of gemcitabine when combined with pretargeted RIT in nude mice with human colon cancer xenografts.[11] In that study, mice bearing 100-200 mm^3 subcutaneous LS174T tumors were treated with either pretargeted RIT, two intraperitoneal injections of gemcitabine at 50-200 mg/kg one day before and one day after administration of ^{131}I-labeled peptide, or combined modality treatment. Whereas both pretargeted RIT and gemcitabine given as monotherapies did not result in significant growth delay of the subcutaneous tumors, the combination of both treatment modalities resulted in a highly significant delay of tumor growth, which was dependent on the dose of gemcitabine used.

In the present study gemcitabine was very toxic in both dosing regimens tested. The dose levels chosen in both MTD studies were based on the doses utilized in the above-mentioned studies in which gemcitabine was combined with RIT. Cardillo et al.[5] and Gold et al.[7] administered gemcitabine intraperitoneally every third day for a total of five administrations at 2 mg/mouse and reported a maximum mean weight loss of only 4.5 ± 5.5% on day 7. In the present study, however, one out of six mice treated at 1 mg/mouse died of gemcitabine-related toxicity two days after the first administration, whereas of the six mice treated at 3 mg/mouse four died of acute treatment-related toxicity. Therefore, the MTD of gemcitabine administered every third day for a total of four administrations was set at <1.0 mg/mouse. Since once-weekly administrations of up to 6 mg/mouse have been reported feasible,[6] the MTD of gemcitabine when administered daily for five consecutive days was sought between 0.022 mg and 0.6 mg per mouse per administration. Gemcitabine, however, was lethal in the majority of mice treated at 0.066 mg/administration and in all mice treated at higher doses. Hence, the MTD of daily administrations of gemcitabine on five consecutive days was set at only 22 μg/mouse/administration. The differences between the MTDs

found in the present studies and those reported by other authors might be attributed to differences in mouse strains.

The most efficacious dosing regimen of gemcitabine to fully exploit its radiosensitizing effect is still a matter of investigation in both preclinical and clinical studies. From preclinical studies on the combination of gemcitabine and external beam radiotherapy (RT), it became clear that the effects of combination therapy become synergistic when gemcitabine treatment precedes RT.[21] In vitro studies indicated that a brief exposure (2 hours) of HT-29 colon cancer cells to gemcitabine resulted in radiosensitization for up to 48 hours.[22] In RIT the radiation energy is delivered to the tumors over a prolonged period of time. Furthermore, since in a previous study we showed that the uptake of radioiodinated MN-14 in the small intraperitoneal tumor deposits is more than 50% ID/g for up to 72 hours p.i.[12], we assumed that, similar to the administration regimen reported by the group of Cardillo and Gold, repeated administrations of gemcitabine starting concurrently with RIT, might effectively sensitize the intraperitoneal tumors to RIT. In contrast to the synergistic effects of gemcitabine when combined with RIT as reported by other authors, gemcitabine did not enhance the efficacy of RIT in the present studies. When administered every third day for a total of four administrations, gemcitabine monotherapy still had a modest, albeit significant antitumor effect, which was dose-dependent. When combined with RIT at this dosing schedule, median survival of the combination therapy groups improved relative to the RIT monotherapy group, although the differences did not reach statistical significance. In the second therapy study, daily administrations of gemcitabine at 0.022 mg/mouse/administration for five consecutive days, which was considered the MTD of this administration regimen, did not affect survival, nor did it enhance the efficacy of RIT.

In conclusion, when given concurrently with RIT either every third day for a total of four administrations or daily for a total of five consecutive days, gemcitabine did not enhance the therapeutic efficacy of RIT at the dose regimens employed.

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7

Radioimmunotherapy is an effective adjuvant treatment modality after cytoreductive surgery of experimental peritoneal carcinomatosis of colonic origin

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Next to the lymphatic and hematogenous routes of dissemination, colorectal cancer can give rise to peritoneal spread of cancer cells. This so-called seeding of cancer cells may lead to peritoneal carcinomatosis, a disease characterized by the presence of multiple tumorous implants on the peritoneum. Although hematogenous dissemination forms the greatest threat to patients with colorectal cancer, it has been estimated that peritoneal carcinomatosis dominates the clinical picture in one out of four patients with recurrent colorectal cancer.[1-3] In the past two decades peritoneal carcinomatosis of colorectal origin is increasingly being recognized as a distinct clinical entity, which should not necessarily be regarded as generalized disease but, similar to liver metastases, may rather be one of the first steps of dissemination.[4] For this reason the efficacy of cytoreductive surgery and intraperitoneal chemotherapy, aimed at locoregional, i.e. intraperitoneal control, has been subject of investigation in a dozen medical centers worldwide.[5] The results published to date indicate that this aggressive approach is effective and may indeed result in improved survival compared to strictly palliative chemotherapy and surgery when needed in selected groups of patients.[6,7] The completeness of resection as well as the tumor load were consistently shown to be the most important factors predictive of long-term survival. Still, after complete resection of all macrosopic disease and adjuvant intraperitoneal chemotherapy the reported five-year survival rate varies from 20% till 54% with the majority of recurrences occurring intraperitoneally.[6,8,9] More effective adjuvant treatment modalities are therefore necessary to improve the results of cytoreductive surgery.

Radioimmunotherapy using radiolabeled monoclonal antibodies directed against tumor-associated antigens offers the opportunity to selectively irradiate tumor cells, while sparing normal tissues. Several preclinical studies, including animal models of liver metastases or peritoneal carcinomatosis of colorectal origin, have indicated that radioimmunotherapy can be very effective and possibly even superior to chemotherapy.[10-12] Since tumor targeting with radiolabeled monoclonal antibodies is more efficient in small volume disease, radioimmunotherapy is considered most suitable for minimal or microscopic residual disease.[13] In this regard, the results of a recently published phase II trial investigating the efficacy of adjuvant radioimmunotherapy using the ^{131}I-labeled anti-CEA MAb hMN-14 after salvage resection of liver metastases of colorectal origin, suggested that radioimmunotherapy may indeed improve both overall median survival and five-year survival rate, compared to historical and contemporaneous controls.[14]

The MG1 MAb, raised against the syngeneic rat colon carcinoma cell line CC-531, has been shown to localize to in vivo growing CC-531 tumors in Wag/rij rats.[15,16] We hypothesized that radioimmunotherapy using radiolabeled MG1 might be an effective adjuvant treatment modality after cytoreductive surgery of peritoneal carcinomatosis. To test this hypothesis, first the biodistribution of MG1 labeled with ^{125}I or ^{111}In was determined in Wag/Rij rats with small volume peritoneal CC-531 carcinomatosis. Subsequently, the efficacy of radioimmunotherapy using ^{177}Lu-labeled MG1 was ascertained. Finally, the efficacy of cytoreductive surgery followed by adjuvant radioim-

munotherapy using ^{177}Lu-labeled MG1 was assessed and compared with that of exploratory laparotomy followed by radioimmunotherapy, cytoreductive surgery only, or exploratory laparotomy only.

Materials and methods

Animals

Male Wag/Rij-rats, ten to twelve weeks old with a mean weight of 245 ± 8 g were obtained from Charles River Laboratories (Sulzfeld, Germany). Rats were accustomed to laboratory conditions for one week prior to experimental use and housed under nonsterile, standard laboratory conditions (temperature, 20–24°C; relative humidity, 50-60%; 12 h light/12 h dark) on sawdust in filter-topped cages (two rats per cage) with free access to animal chow (Snif Voer, Soest, The Netherlands) and water. All experiments were conducted in accordance with the principles laid out by the revised Dutch Act on Animal Experimentation (1997) and approved by the institutional Animal Welfare Committee of the Radboud University Nijmegen.

Cell line

The syngeneic rat colon carcinoma cell line CC-531, derived from colon tumors of Wag/Rij rats exposed to 1,2-dimethyl-hydrazine, was used.[17] CC-531 was cultured and maintained as monolayers on plastic in Dulbecco's Modified Eagle Medium (DMEM, GIBCO, BRL Life Sciences Technologies, The Netherlands) supplemented with 10% fetal calf serum (GIBCO), 2 mM L-glutamine, penicillin (100 units/mL) and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere with 5% CO_2. Prior to inoculation, tumor cells were washed with phosphate-buffered saline (PBS), harvested with 0.25% trypsin (3 min at 37 °C), resuspended in PBS, spun down (5 min at 700 x *g*), counted and resuspended once again in PBS to the appropriate concentration.

Monoclonal antibody

The MG1 MAb was purchased from Antibodies for Research Applications BV (Gouda, The Netherlands, www.abforresearch.nl). The MG1 MAb is a murine IgG_{2a} antibody, raised by immunization of mice with CC-531 colon carcinoma cells.[15,16,18] It recognizes a cell surface structure of about 80 kDa and localizes to tumor cells when injected in rats bearing CC-531 tumors with minimal cross reactivity to other cell types. Purity was checked by SDS-PAGE under nonreduced conditions and by means of fast protein liquid chromatography (FPLC) on a Phenomenex Biosep 3000 column, eluted with phosphate buffered saline (PBS, pH 7.2, 1 mL/min).

Radioiodination

Antibodies were radioiodinated with ^{125}I (Amersham, Den Bosch, The Netherlands) using the iodogen-method, as described previously.[19] Briefly, antibodies (20 μg) and ^{125}I (4.07 MBq) were incubated at room temperature in 80 μl of PBS (0.10 M, pH 7.4) in a glass vial, coated with 50 μg iodogen. After twelve minutes, the reaction mixture was separated on a PD-10 column (Amersham Biosciences, Uppsala, Sweden), eluted with PBS, 0.5% bovine serum albumin (BSA). Labeling efficiency of the radioiodination reaction was 42%. The specific activity of ^{125}I-MG1 was 85 kBq/μg.

^{111}In- and ^{177}Lu-labeling

^{111}In and ^{177}Lu (specific activity 862 GBq/mg in the radioimmunotherapy monotherapy study, and 982 GBq/mg in the cytoreductive surgery + radioimmunotherapy study, respectively) was purchased from Tyco Mallinckrodt Medical BV (Petten, The Netherlands) and IDB Holland BV (Baarle Nassau, The Netherlands), respectively. ^{111}In- and ^{177}Lu-labeling was performed under strict metal-free conditions. To allow labeling of the antibodies with ^{111}In or ^{177}Lu, the MG1 MAb was conjugated with isothiocyanatobenzyl-diethylenetriaminepentaacetic acid (ITC-DTPA, Macrocyclics, Dallas, TX). Briefly, ITC-DTPA was conjugated to MG1 in a 0.1 M carbonate buffer, pH 9.0 using a 100-fold molar excess of ITC-DTPA as described by Ruegg et al.[20] with minor modifications (conjugation period of one hour at room temperature). The DTPA-MG1 immunoconjugate was then purified by extensive dialysis against 0.1 M ammonium acetate buffer, pH 5.4. The number of DTPA ligands per antibody molecule was determined according to the method described by Hnatowich et al.[21] The purified DTPA-MG1 conjugate (DTPA/MG1 ratio 1.6 : 1, 1.0 mg/mL) was incubated with ^{111}In or ^{177}Lu in 0.1 M ammonium acetate buffer, pH 5.4 at room temperature (60 minutes). Labeling efficiency of both labeling procedures exceeded 98%. Specific activity of the ^{111}In-DTPA-MG1 preparation (hereafter referrend to as ^{111}In-MG1) used in the biodistribution study was 1.1 MBq/μg. Specific activities of the ^{177}Lu-DTPA-MG1 preparations (hereafter referrend to as ^{177}Lu-MG1) used in the radioimmunotherapy monotherapy study and the cytoreductive surgery + radioimmunotherapy study was 0.81 MBq/μg and 0.89 MBq/μg, respectively. Since ^{177}Lu is a bone-seeking radionuclide, after completion of the labeling procedure 1 mM EDTA was added to the solutions to scavenge the remaining free ^{177}Lu ions.[22]

Quality control of the radiolabeled preparations

All reaction mixtures were purified on a PD-10 column, eluted with PBS, 0.5% BSA. Radiochemical purity (RCP) was determined using instant thin-layer chromatography (ITLC) on silicagel strips (Gelman Sciences, Ann Arbor, MI) using 0.10 M citrate buffer

(pH 6.0) as the mobile phase. The RCP of the ^{125}I-, ^{111}In- and ^{177}Lu-MG1 preparation was 98%, 99% and 99%, respectively.
The immunoreactivity of the radiolabeled MG1 preparations was essentially determined as described by Lindmo et al.[23] Briefly, a fixed amount of labeled antibody (10,000 cpm) was incubated (6 hours, 37 °C) with increasing concentrations of CC-531 tumor cells (1.2 x 10^6 – 20 x 10^6 cells/mL) in 0.5 mL binding buffer (RPMI medium containing 0.5% BSA and 0.05% NaN_3). A duplicate of the lowest cell concentration was incubated in the presence of an excess unlabeled antibody to correct for non-specific binding. After six hours of incubation at 37°C, the cells were spun down (500 g, 5 min) and activity in the pellet was determined in a well-type gamma-counter. The inverse of the tumor cell bound fraction was plotted against the inverse of the cell concentration and the immunoreactive fraction (IRF) was calculated from the y-axis intercept. Immunoreactivity of each of the radiolabeled MG1 preparations exceeded 65%. Radiolabeled antibody preparations were administered within two hours after the labeling procedures.

Animal model of peritoneal carcinomatosis

The animal model of peritoneal carcinomatosis using CC-531 has been described previously.[16] Briefly, peritoneal carcinomatosis was induced by intraperitoneal inoculation of 2.0 – 5.0 x 10^6 CC-531 tumor cells, suspended in 2.5 mL PBS in a 5.0 mL syringe using a 18 gauge needle. In this model, the first macroscopic tumor deposits are seen 4 to 7 days after tumor cell inoculation. Three to five weeks after tumor induction, bulky peritoneal carcinomatosis with hemorrhagic ascites is present.

Biodistribution of ^{125}I-/^{111}In-labeled MG1

We previously demonstrated in a nude mouse model of peritoneal carcinomatosis of colonic origin that ^{131}I and ^{177}Lu are the most suitable radionuclides for radioimmunotherapy of small peritoneal metastases.[24] To determine the most suitable radionuclide for radioimmunotherapy in the current model, the biodistribution of MG1 labeled with either ^{125}I or ^{111}In, as surrogate radionuclides for ^{131}I and ^{177}Lu, respectively, was determined. Eight days after intraperitoneal inoculation of 5.0 x 10^6 CC-531 tumor cells, five rats received an intraperitoneal injection of 370 kBq ^{125}I-MG1 and 370 kBq ^{111}In-MG1 (total MG1 protein dose 5 µg per rat), solubilized in 1 mL PBS, BSA 0.5%. Three days after injection of the radiolabeled antibodies, the rats were euthanized using O_2/CO_2-asphyxiation and dissected. At dissection tumor, blood, bone marrow, liver, spleen, kidney, intestine, lung, muscle, skin and thymus were sampled, blotted dry, and immediately weighed. Activity was measured in a shielded well-type gamma-counter (Wizard, Pharmacia-LKB, Sweden). To correct for physical decay and to calculate uptake of the radiolabeled antibody in each sample as a fraction of the injected

dose, aliquots of the injected dose were counted simultaneously. The results were expressed as percentage of the injected dose per gram tissue (% ID/g).

The efficacy of radioimmunotherapy using ^{177}Lu-MG1

Before investigating the efficacy of adjuvant radioimmunotherapy after cytoreductive surgery, first the efficacy of radioimmunotherapy given as monotherapy was assessed. Four days after intraperitoneal inoculation of 2.0 x 10^6 CC-531 tumor cells, rats were randomly assigned to be treated with radioimmunotherapy (74 MBq ^{177}Lu-MG1, 91 μg per rat, solubilized in PBS, 0.5% BSA, 1 mM EDTA), unlabeled MG1 (91 μg, solubilized in PBS, 0.5% BSA, 1 mM EDTA) or the carrier (PBS, 0.5% BSA, 1 mM EDTA) (8-10 rats per group). In addition, an extra group of five rats received tracer doses of ^{177}Lu-MG1 (3.7 MBq per rat) to determine the biodistribution at 72 hours post-injection (p.i.). Since ^{177}Lu is a bone-seeking radionuclide, uptake in femur was determined as well. The radiolabeled preparation used to determine the biodistribution was augmented with unlabeled DTPA-MG1 to the same total protein dose of 91 μg per rat, as used in the rats that were given radioimmunotherapy. Thirty days after tumor cell inoculation, rats were euthanized and dissected. At dissection, all tumor nodules were excised and weighed.

The effect of cytoreductive surgery with or without adjuvant radioimmunotherapy

Fourteen days after intraperitoneal inoculation of 2.0 x 10^6 CC-531 tumor cells, 35 rats were randomly assigned to undergo cytoreductive surgery (n=18) or exploratory laparotomy (n=17). Subsequently, all rats underwent a midline laparotomy during which the intraperitoneal tumor load was semiquantitatively scored (see below). After completion of the surgical procedures, those rats that had undergone cytoreductive surgery were distributed into two groups by an independent nuclear medicine physician (WJGO), after stratification for tumor load, completeness of resection and whether or not a splenectomy had been performed. Similarly, those rats that had undergone exploratory laparotomy only were divided into two groups after stratification for tumor load. Three days after surgery, one group of rats that had been treated with cytoreductive surgery and one group of rats that undergone exploratory laparotomy only received an intraperitoneal injection of 56 MBq ^{177}Lu-MG1 (total MG1 protein dose 63 μg). The remaining rats received intraperitoneal injections of the carrier (PBS, 0.5% BSA, 1 mM EDTA). Thus, four treatment groups were created: 1. exploratory laparotomy only (n=9); 2. exploratory laparotomy + radioimmunotherapy (n=8); 3. cytoreductive surgery only (n=9); 4. cytoreductive surgery + radioimmunotherapy (n=9).

Operative procedures

The surgical procedures were carried out under clean conditions. One hour prior to operation, rats received buprenorphine subcutaneously (5 μg, 0.1 mL) for analgesia. Immediately after the onset of general anesthesia (isoflurane/O_2/N_2O), the abdomen was shaved and disinfected with chlorohexidine/ethanol. During the operation, rats were placed on a warmed mattress to limit heat loss. Rats subsequently underwent a 7.5 cm midline laparotomy from the xyphoidal process till just above the penis. After opening the abdomen, the abdominal cavity was carefully inspected, starting with the upper abdomen, including the greater omentum, the liver hylum, the perisplenic region and the diaphragm. Subsequently, the gonadal fat pads and the kidneys were inspected. Finally, the intestines were gently lifted out of the abdomen, enabling inspection of the complete mesentery and Douglas' pouch. Tumor growth at each of these sites was scored 0, 1, 2, or 3, where 0 indicated no macroscopic tumor growth, 1 meant little tumor growth, 2 indicated moderate tumor growth, and 3 indicating abundant tumor growth. The sum of the tumor scores of all sites represented the peritoneal cancer index (PCI).
In those rats assigned to undergo cytoreductive surgery, it was then attempted to resect all macroscopic tumor deposits (MJK). In case of irresectable disease, tumor nodules were fulgurated using an electrocoagulation device. The resected tumor nodules, including the omentectomy specimens, were collected and weighed. When the surgical cytoreduction was considered maximal, the muscular layer of the abdomen was closed by continuous Vicryl 3/0 sutures. The skin was closed by iron wound autoclips. 10 mL of warmed saline was injected subcutaneously for rehydration. Twelve hours postoperatively, and once-daily on the second and third day postoperatively, rats once again received subcutaneous injections of buprenorphine (5 μg, 0.1 mL).

Follow-up

The primary endpoint was survival. Body weight was measured daily in the first 14 days after treatment and three times weekly thereafter as measure of toxicity. Rats were monitored daily until the humane endpoint had been reached, as determined by one single experienced and independent animal technician, who was blinded to the therapeutic regimen. At the time of the humane endpoint, rats were usually lethargic, showing signs of advanced peritoneal carcinomatosis, such as the presence of ascites or bulky intraperitoneal tumor growth, and were expected to die within one or two days. When the humane endpoint had been reached, rats were euthanized by O_2/CO_2-asphyxiation and immediately dissected. At dissection, intraperitoneal tumor growth was semi-quantitatively scored as described above. After calculating a PCI, all macroscopic tumor deposits were excised and weighed. The experiment was terminated at 118 days after tumor cell inoculation when the remaining rats were euthanized and dissected. In case of absence of macroscopic tumor growth, relevant organs, including

the greater omentum, the mesentery and the diaphragm were removed for routine histopathological hemotoxilin & eosin (H&E) and/or immunohistochemical staining using the murine MG1 antibody and a horse-anti-mouse IgG antibody (Vector Laboratories Inc., Burlingame, CA, USA).

Statistical analysis

Data are expressed as means ± standard deviation (SD) unless stated otherwise. Statistical analysis was performed by means of the GraphPad Prism 4.00 software (GraphPad Software, San Diego, CA, USA). Single comparisons were analyzed using the paired t-test or the non-parametric Mann Whitney *U* test. Multiple comparisons were analyzed using the one-way ANOVA test. Bonferroni correction for multiple testing was applied. Survival curves were compared using the Log-rank test. All tests were two-sided; the level of statistical significance was set at a *P* value of <0.05.

Results

Biodistribution of ^{125}I-/^{111}In-labeled MG1

To assess the most suitable radionuclide for radioimmunotherapy, the biodistribution of ^{125}I-MG1 and ^{111}In- MG1 was determined 72 hours post-injection. The results are summarized in Figure 1. There was a preferential uptake of both radiolabeled antibody preparations in the tumor, although uptake of ^{125}I-MG1 in tumor tissue was not significantly higher than the corresponding blood levels (P=0.093). Uptake of ^{111}In-MG1 in tumor tissue was higher than that of ^{125}I-MG1, although the difference was not statistically significant (4.1 ± 2.3% ID/g vs 1.1 ± 0.5% ID/g, P=0.053). Tumor-to-blood and tumor-to-bone marrow ratios were significantly higher than those obtained for ^{125}I-MG1 (9.2 ± 5.3 vs. 2.4 ± 1.4 (P=0.040) and 19.5 ± 8.4 vs. 7.6 ± 4.1 (P=0.036), respectively. Likewise, uptake of ^{111}In-MG1 in spleen and liver was higher than that of ^{125}I-MG1, although the difference for the latter organ did not reach statistical significance. Based on the favorable biodistribution of ^{111}In-MG1, it was decided to use ^{177}Lu in the radioimmunotherapy studies.

Efficacy of radioimmunotherapy using ^{177}Lu-MG1

To investigate the therapeutic potential of radioimmunotherapy using ^{177}Lu-labeled MG1, rats with small peritoneal metastases were treated with either ^{177}Lu-MG1 (74 MBq/rat), unlabeled DTPA-MG1, or the carrier. In addition, the biodistribution of ^{177}Lu-MG1 was determined. Mean body weight of the control rats as well as that of the rats treated with DTPA-MG1 gradually increased during the course of the study.

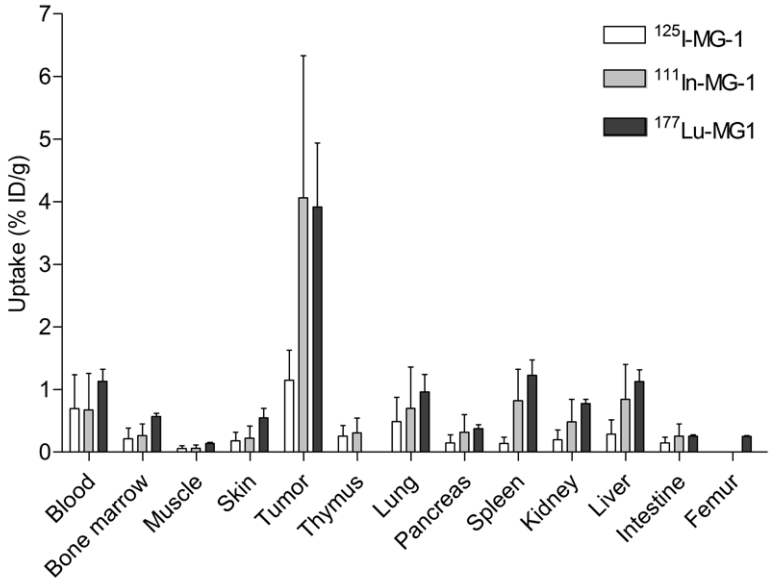


Figure 1
Biodistribution of ^{125}I-, ^{111}In, or ^{177}Lu-labeled MG1 in Wag/Rij rats with small peritoneal CC-531 metastases. Note that the biodistribution of ^{177}Lu-MG1 was determined in a separate experiment.

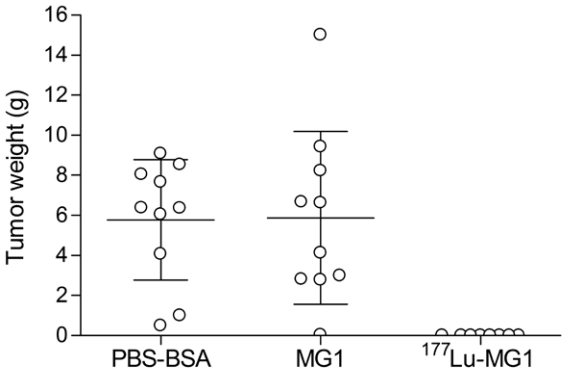


Figure 2
Tumor weight at dissection of Wag/Rij rats with small peritoneal CC-531 metastases 26 days after treatment with either PBS, 0.5% BSA (controls), unlabeled MG1 or ^{177}Lu-MG1.

Mean body weight of the rats treated with 74 MBq ^{177}Lu-MG1, however, decreased to a maximum mean body weight loss of 2.6 ± 3.2% on day 5 and 6 after intraperitoneal administration, after which all rats regained body weight again (data not shown).
The biodistribution of ^{177}Lu-MG1 at 72 hours p.i. was similar to that of ^{111}In-MG1 (Figure 1). Mean uptake in tumor tissue was 3.9 ± 1.0% ID/g, whereas the tumor-to-blood and tumor-to-bone marrow ratios were 3.5 ± 0.2 and 7.0 ± 1.2, respectively. Uptake in femur was only 0.25 ± 0.02% ID/g.

Thirty days after intraperitoneal tumor cell inoculation the rats were euthanized. At dissection the majority of the rats that had received PBS, 0.5% BSA, 1mM EDTA (controls) or unlabeled DTPA-MG1 had abundant intraperitoneal tumor growth with hemorrhagic ascites. In contrast, the rats that had been treated with 74 MBq ^{177}Lu-MG1 had very little tumor growth. Mean tumor weight of the control rats and the rats treated with unlabeled ITC-DTPA-MG1 was practically identical, 5.8 ± 3.0 g and 5.9 ± 4.3 g, respectively. In contrast, mean tumor weight of the rats that had been treated with radioimmunotherapy was 0.00 ± 0.00 g ($P<0.01$ comparing radioimmunotherapy with either PBS, 0.5% BSA or unlabeled MG1; Figure 2).

The effect of cytoreductive surgery with or without adjuvant radioimmunotherapy

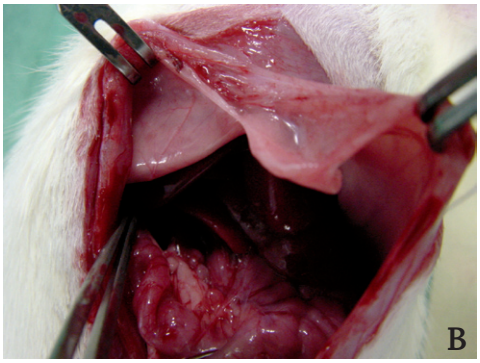
To assess the efficacy of radioimmunotherapy when given as adjuvant treatment after cytoreductive surgery, rats with small peritoneal metastases were treated with cytoreductive surgery only, exploratory laparotomy + radioimmunotherapy, or cytoreductive surgery + radioimmunotherapy. Control rats underwent exploratory laparotomy only.

Tumor load at laparotomy

The greater omentum was the most common site of tumor growth (all rats), followed by the liver hylum (34 out of 35 rats), the mesentery (30 out of 35 rats) and the perisplenic region (25 out of 35 rats). Fifteen rats had tumor growth on the parietal peritoneum at the site of tumor cell inoculation. Macroscopic tumor growth in the gonadal fatpads was observed in eight rats, at the diaphragm in one rat, near the kidneys in one rat, whereas none of the rats had developed macroscopic tumor growth in Douglas' pouch. The diameter of the tumor nodules was always lower than 5 mm and ranged from 1 to 4 mm. None of the rats had malignant ascites. Median PCI of all the rats was 7 (range 3-10). In Figure 3 three representative examples of macroscopic tumor growth at the greater omentum, the liver hylum and the mesentery are depicted.

Resections

In the eighteen rats assigned to undergo cytoreductive surgery an omentectomy was routinely carried out. Omentectomy involved removing as much of the omental tissue as posssible, from the hilum of the spleen (conserving the splenic artery arcade), from the greater curvature of the stomach (removing the epiploic arcade) and from the retrogastric extension of the omental leaf. Perisplenic tumor growth necessitated splenectomy in nine out of eighteen rats that underwent cytoreductive surgery. In fifteen out of eighteen rats undergoing cytoreductive surgery, the resection of all tumor nodules seemed macroscopically complete, whereas in three rats macroscopic disease was left behind. In those three rats that had undergone an incomplete cytoreduction,



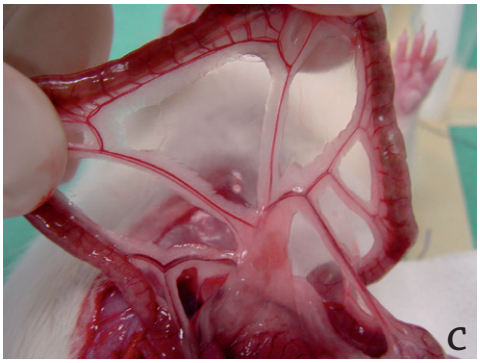


Figure 3
Macroscopic appearance of intraperitoneally growing CC-531 tumors in Wag/Rij rats. A, multiple tumor deposits in the greater omentum; B, two tumor deposits in the liver hylum; C, one tumor deposit on the mesentery.

the size of the residual disease was always less than 1 mm. The mean weight of the resection specimens was 0.21 ± 0.07 gram.

Assignment to (adjuvant) radioimmunotherapy.
After stratification for PCI, completeness of resection and whether a splenectomy had been or would have been carried out, seventeen rats (nine rats that had undergone cytoreductive surgery and eight rats that had undergone exploratory laparotomy), received an intraperitoneal injection of 56 MBq ^{177}Lu-MG1, whereas the remaining rats received the carrier (PBS, BSA 0.5%, EDTA 1mM). The four treatment groups were well-balanced with regard to the above-mentioned stratification factors, as well as body weight, the number of tumor nodules per site (data not shown) and, when applicable, the weight of the resection specimens (Table 1).

Toxicity
The relative body weight, expressed as the percentage of the body weight on the day of surgery, is depicted in Figure 4. On the third postoperative day, prior to the administration of radioimmunotherapy, those rats that were treated with cytoreductive sur-

Table 1. Rat-, surgical and pathological characteristics

	EL only	EL + RIT	CS only	CS + RIT
Body weight (g)	275 ± 8	271 ± 7	273 ± 8	274 ± 11
Tumor score per site†				
Subcutaneously	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Greater omentum	2 (1-3)	2 (1-3)	2 (2-3)	2 (2-3)
Liver hylum	2 (1-2)	2 (1-2)	2 (1-2)	2 (0-2)
Perisplenic	1 (0-2)	1 (0-1)	1 (0-2)	1 (0-2)
Mesentery	1 (0-2)	1 (0-2)	1 (1-2)	1 (0-2)
Gonadal fatpads	0 (0-1)	0 (0-1)	0 (0-1)	0 (0-1)
Diaphragm	0 (0-1)	0 (0-0)	0 (0-0)	0 (0-0)
Subrenal	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-1)
Parietal peritoneum	0 (0-1)	0 (0-1)	0 (0-1)	1 (0-1)
Douglas' pouch	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Total	6 (4-8)	7 (3-8)	6 (5-10)	7 (5-10)
Weight resection specimen (g)	NA	NA	0.21 ± 0.09	0.22 ± 0.06
Splenectomy				
Yes	NA	NA	4	5
No	NA	NA	5	4
Resection macroscopically complete				
Yes	NA	NA	8	7
No	NA	NA	1	2

EL, Exploratory laparotomy; CS, cytoreductive surgery; RIT, Radioimmunotherapy; NA, Not applicable. † Tumor score is expressed as median (range)

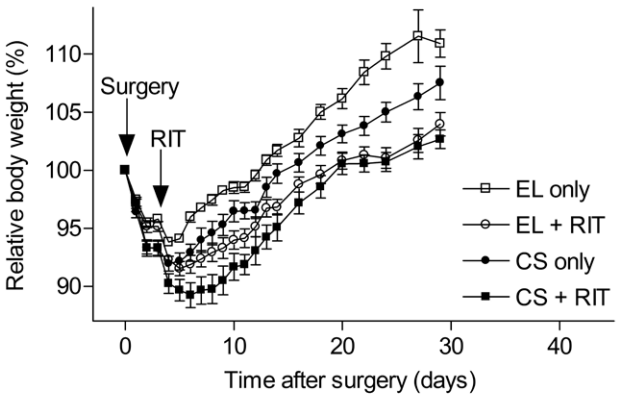


Figure 4
The relative body weight of Wag/Rij rats with small peritoneal CC-531 metastases in the first month after exploratory laparotomy (EL) only, EL + radioimmunotherapy (RIT), cytoreductive surgery (CS) only, or CS + RIT. Data represent means ± standard error of the mean (SEM).

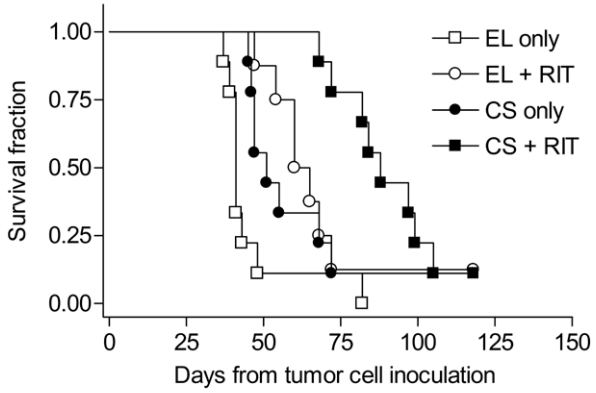


Figure 5
Survival curves for Wag/Rij rats with small peritoneal CC-531 metastases after exploratory laparotomy (EL) only, EL + radioimmunotherapy (RIT), cytoreductive surgery (CS) only, or CS + RIT. There was a highly significant trend towards improved survival for the rats treated with CS + RIT as compared to both CS only and EL + RIT (P=0.0004 for both trend analyses).

gery had lost more body weight than those that underwent exploratory laparotomy (6.7 ± 1.9% vs 4.5 ± 1.1%, P=0.0002). Maximum mean body weight loss of the rats of the exploratory laparotomy only group was 6.4 ± 0.6% four days after the surgery. Maximum body weight loss of the rats of the exploratory laparotomy + radioimmunotherapy group amounted 8.5 ± 1.7% five days after surgery or two days after start of the radioimmunotherapy (P=0.001 compared to the maximum weight loss of the rats of the exploratory laparotomy only group). Maximum body weight loss of the rats of the cytoreductive surgery only group amounted 8.1 ± 1.9% four days after surgery (P=0.015 compared to the exploratory laparotomy only group; P=0.888 compared to the exploratory laparotomy + radioimmunotherapy group). Maximum mean body weight loss of the rats of the cytoreductive surgery + radioimmunotherapy group was

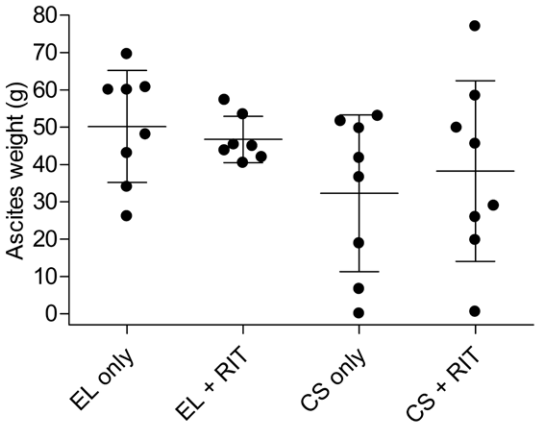


Figure 6a
Ascites weight found in Wag/Rij rats with small peritoneal CC-531 metastases at the time of death after exploratory laparotomy (EL) only, EL + radioimmunotherapy (RIT), cytoreductive surgery (CS) only, or CS + RIT. Differences were not statistically significant (P=0.2219).

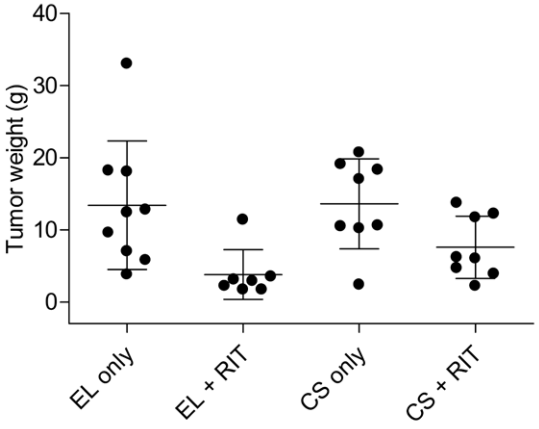


Figure 6b
Tumor weight found in Wag/Rij rats with small peritoneal CC-531 metastases at the time of death after exploratory laparotomy (EL) only, EL + radioimmunotherapy (RIT), cytoreductive surgery (CS) only, or CS + RIT. Tumor weight of the rats treated with EL + RIT was significantly lower as compared to that obtained in rats treated with EL only or CS only (P<0.05).

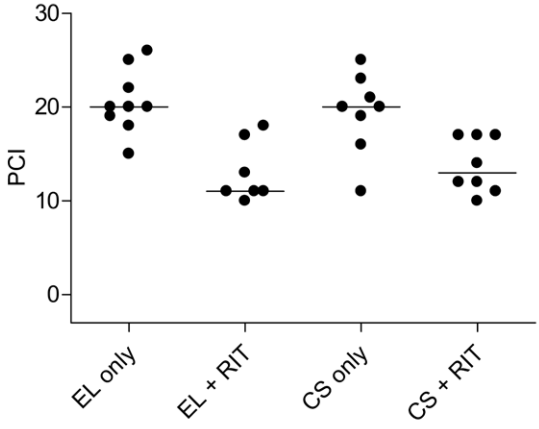


Figure 6c
PCI found in Wag/Rij rats with small peritoneal CC-531 metastases at the time of death after exploratory laparotomy (EL) only, EL + radioimmunotherapy (RIT), cytoreductive surgery (CS) only, or CS + RIT. The PCI of the rats treated with RIT, preceded by either EL or CS, was significantly lower as compared to that obtained in rats treated with EL only or CS only (P<0.05). Lines indicate medians.

10.7 ± 3.3% six day after surgery or three days after start of the radioimmunotherapy (P=0.0003 compared to the exploratory laparotomy only group; P=0.200 compared to the exploratory laparotomy + radioimmunotherapy group; P=0.136 compared to the cytoreductive surgery only group. After reaching their nadirs, rats regained body weight again at approximately the same rate.

Survival

Thirty-two rats died due to intraperitoneal tumor growth. The survival curves of the various treatment groups are shown in Figure 5. Median survival of the control rats that underwent exploratory laparotomy only was 41 days (range 37-82). Median survival of the rats that had undergone cytoreductive surgery only amounted 51 days (45-118) (P=0.0544 when compared to the control rats). Median survival of the rats that were treated with exploratory laparotomy followed by intraperitoneal radioimmunotherapy was 61.5 days (47-118) (P=0.0304 when compared to the control rats; P=0.47 when compared to the cytoreductive surgery only group). Cytoreductive surgery + radioimmunotherapy resulted in the highest median survival of 88 days (68-118) (P=0.0001 when compared to the control rats; P=0.0503 when compared to the cytoreductive surgery only group; P=0.0822 when compared to the exploratory laparotomy + radioimmunotherapy group). There was a highly significant trend toward improved survival in the cytoreductive surgery + radioimmunotherapy group, both when compared to the cytoreductive surgery only group as well as the exploratory laparotomy + radioimmunotherapy group (P=0.0004 for both trend analyses).

Observations at dissection

Formation of hemorrhagic ascites was the most common cause of death. Of the 32 rats that died due to intraperitoneal tumor growth, 31 had hemorrhagic ascites. In Figure 6a-c the ascites weight, tumor weight and PCI found at dissection are depicted. Mean ascites weight varied from 32.2 ± 21.0 g in the rats that had undergone cytoreductive surgery only to 50.2 ± 15.0 g in the control group (P=0.22). Mean tumor weight found at dissection at the time rats reached their humane endpoints varied from 3.8 ± 3.4 g in the exploratory laparotomy + radioimmunotherapy group to 13.6 ± 6.2 g in the cytoreductive surgery only group (P=0.011). Median PCI varied from 11 (range 10-18) in the exploratory laparotomy + radioimmunotherapy group to 20 (18-26) in the cytoreductive surgery only group (P=0.0002). Multiple comparison analyses indicated that the median PCI of the exploratory laparotomy + radioimmunotherapy and cytoreductive surgery + radioimmunotherapy was significantly lower when compared to both the exploratory laparotomy only as well as the cytoreductive surgery only groups. There was a highly significant correlation between the PCI and the weight of the dissected tumor deposits (Spearman r=0.8283, P<0.0001). However, there was no correlation between ascites weight and PCI (Spearman r=0.1948, P=0.2670) or tumor weight (Spearman r=-0.0629, P=0.7239).

Long-term survivors

At the end of the experiment (118 days after tumor cell inoculation), there were three rats (one treated with exploratory laparotomy + radioimmunotherapy; one treated with cytoreductive surgery only; and one treated with cytoreductive surgery + radioimmunotherapy, respectively), without signs of intraperitoneal tumor growth. At dissection, there was no evidence of disease in any of these rats. Histopathological examination of relevant organs, including the diaphragm, the mesentery, and, when available, the greater omentum did not reveal microscopic residual disease in any of these rats.

Discussion

The primary aim of the present experimental studies was to investigate the therapeutic efficacy of radioimmunotherapy when administered postoperatively as adjuvant treatment after surgical cytoreduction of peritoneal carcinomatosis of colonic origin. Whereas the median survival following cytoreductive surgery only and exploratory laparotomy + radioimmunotherapy was only 10 days (P=0.05) and 21.5 days (P=0.03) longer than that of the control rats that had undergone exploratory laparotomy only, the combination of cytoreductive surgery and radioimmunotherapy resulted in an improvement of 43 days (P=0.0001), which suggests an additive effect of the two treatment modalities.

The syngenic rat colon carcinoma cell line CC-531 was used because of its reproducible *in vivo* growth pattern in Wag/Rij rats. Hagenaars et al. studied the tumor targeting capabilities of the MG1 MAb after intraperitoneal administration in Wag/Rij rats with CC-531 liver metastases and found that the antibody selectively localized to the CC-531 tumors, with some cross-reactivity to thymus, lymph node, salivary glands, and skin.[15,16] However, no data were provided on the absolute or percentual uptake in tumor tissue of MG1. Therefore, before initiating radioimmunotherapy studies, the biodistribution of the MG1 MAb labeled with ^{125}I or ^{111}In, as surrogate radionuclides for ^{131}I and ^{177}Lu, respectively, was studied. Both ^{125}I- and ^{111}In-labeled MG1 preferentially accumulated in the intraperitoneal CC-531 tumors. The uptake of ^{111}In-MG1 in tumor tissue, however, was higher, which is probably due to differences in cellular routing of the radiolabeled metabolites after intracellular catabolization of the antibodies.[25] After internalization by the cancer cell, radiolabeled antibodies are enzymatically degraded and metabolized in the lysosomes. After intralysosomal metabolization of monoclonal antibodies that are radioiodinated by conventional methods, the radioiodinated tyrosine residues are excreted, whereas the catabolic products of monoclonal antibodies labeled with ^{111}In- or ^{177}Lu-DTPA, presumably ^{111}In- or ^{177}Lu-DTPA-lysine metabolites, are trapped within the lysosomes, resulting in a prolonged tumor residence time.[26,27] The higher uptake of ^{111}In-ITC-DTPA-MG1 therefore favored the use of ^{177}Lu instead of ^{131}I in radioimmunotherapy studies. Furthermore, the me-

dium-energy beta-emission of ^{177}Lu and its concomitant maximum penetration range in tissue of 2.5 mm in combination with its physical half-life of six days, make ^{177}Lu an attractive radionuclide for radioimmunotherapy of small volume disease, as indeed reported in preclinical and clinical studies.[24,28]

Being an IgG_{2a} antibody, MG1, theoretically, could have an antitumor effect itself through complement-mediated tumor cell lysis or complement- or antibody-dependent cellular cytotoxicity (ADCC). In this regard, Gelderman et al. studied the complement activation potential of the MG1-4 MAb family, and found that MG1 was not able to activate the complement system ***in vitro***.[29] These findings corroborate with the results of the first therapy study reported in the present paper, in which unlabeled MG1 had no measurable antitumor effect in rats with CC-531 peritoneal metastases. radioimmunotherapy using ^{177}Lu-MG1 was given at an activity dose of 74 MBq per rat. Since we previously demonstrated that the maximal tolerated activity dose (MTD) of ^{177}Lu-labeled IgG in 25 g nude mice is 16.7 MBq,[24] the MTD of ^{177}Lu-labeled IgG in 250 g rats could theoretically be as high as 167 MBq. Indeed, radioimmunotherapy using 74 MBq ^{177}Lu-MG1 resulted in minor toxicity, as evidenced by a maximum mean body weight loss of only 2.6 ± 3.2%, which suggests that higher activity dose could safely be given. Still, radioimmunotherapy using 74 MBq ^{177}Lu-labeled MG1 almost completely ablated tumor growth, when given as sole treatment in unoperated rats.

In the therapy studies, cytoreductive surgery consisted of complete/median laparotomy and resection of all macroscopic disease, including formal omentectomy, as is practiced in patients undergoing cytoreductive surgery followed by intraperitoneal chemotherapy.[7] The modest survival benefit (P=0.0504) of the rats treated with cytoreductive surgery only, may partially be explained by the omentectomy, which was routinely carried out in all rats assigned to undergo cytoreductive surgery. In various experimental models it has been demonstrated that tumor cells preferentially accumulate in the so-called milky spots of the omentum in the early stages of intraperitoneal dissemination.[16,30] In a rat model Lawrance et al. showed that omentectomy three to four weeks prior to intraperitoneal or intraluminal tumor cell inoculation significantly reduced intra-abdominal tumor growth.[31] Weese et al. studied the influence of the omentum on intraperitoneal tumor growth in a rat model of peritoneal carcinomatosis of colonic origin and found that omentectomy reduced the incidence of small bowel obstruction by more than 50%.[32] Thus the omentum has been implicated as an initial site of tumor growth after which secondary seeding occurs at sites of peritoneal trauma.

Although cytoreductive surgery and adjuvant intraperitoneal chemotherapy is increasingly being accepted as one of the treatment options for patients with peritoneal carcinomatosis of colorectal origin, there is little experimental data on the efficacy of this treatment. Benoit et al. studied the efficacy of surgical cytoreduction, intraperitoneal chemotherapy using Cisplatin/epinephrine or both treatment modalities in BDIX rats with peritoneal carcinomatosis of colonic origin.[33] Whereas cytoreductive surgery alone did not improve survival, intraperitoneal chemotherapy alone signifi-

cantly improved survival without curing any of the animals. Cytoreductive surgery followed by intraperitoneal chemotherapy, however, cured four out of five animals. Since the uptake of radiolabeled monoclonal antibodies is inversely correlated with tumor size,[13] the efficacy of radioimmunotherapy, theoretically, should be higher in smaller tumor deposits. Therefore, we hypothesized that the combination of cytoreductive surgery followed by radioimmunotherapy would act additively. Indeed, the combination of both treatment modalities resulted in the highest median survival and a highly significant trend towards improved survival, both when compared to cytoreductive surgery only and exploratory laparotomy + radioimmunotherapy. The majority of rats were euthanized because of the development of massive hemorrhagic ascites. Interestingly, whereas the amount of ascites at the time of dissection was similar between the groups, rats treated with radioimmunotherapy, preceded by either exploratory laparotomy or cytoreductive surgery, showed (significantly) less solid tumor growth as compared to those treated with exploratory laparotomy or cytoreductive surgery only. Apparently, radioimmunotherapy effectively inhibited tumor growth but was not able to prevent the formation of ascites.

Conclusion

This is the first experimental study on the efficacy of combined modality treatment including surgery and radioimmunotherapy in an animal model of cancer. Radioimmunotherapy using the ^{177}Lu-labeled MG1 MAb effectively inhibited tumor growth, even at relatively low activity doses. When given postoperatively after cytoreductive surgery in rats with peritoneal carcinomatosis of colonic origin, radioimmunotherapy was associated with improved survival as compared to both cytoreductive surgery and radioimmunotherapy alone. This study provides proof of principle that radioimmunotherapy can be an effective treatment modality when applied as adjuvant treatment modality after resection of tumors with a high risk of recurrence.

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8

Timing of adjuvant radioimmunotherapy after cytoreductive surgery in experimental peritoneal carcinomatosis of colonic origin

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Peritoneal carcinomatosis (PC) of colorectal origin represents an advanced stage of disease, which for long has been regarded as incurable. Until the beginning of the 21st century, patients were usually treated with palliative chemotherapy and surgery when needed. The reported median survival associated with this approach ranges from 5 to 12 months.[1-4] In the nineteeneighties surgical oncologists gained renewed interest in patients with PC, particularly those without hematogenous metastases. Sugarbaker has pioneered a new aggressive approach, consisting of cytoreductive surgery (CS) followed by (hyperthermic) intraperitoneal chemotherapy (HIPEC), aiming at locoregional control of disease.[5] After two decades of phase II trials investigating the feasibility and efficacy of CS followed by HIPEC, Verwaal et al. conducted a phase III trial comparing this aggressive approach to systemic 5-fluorouracil (5-FU) based chemotherapy and surgery when needed and confirmed its superiority.[6] An analysis of the results of 16 clinical trials on the efficacy of CS + HIPEC in patients with PC of colorectal origin, indicated that the extent of carcinomatosis and completeness of resection were the factors most prominently related to survival.[7] Still, five-year survival rates of the patients with the most favorable clinicopathological characteristics varies from only 20% to 53%, with most recurrences occurring intraperitoneally.[8] Therefore, more effective adjuvant treatments are necessary to improve the results of CS.
Although radioimmunotherapy (RIT) using radiolabeled monoclonal antibodies (MAbs) directed against tumor-associated antigens has been accepted as one of the clinically accepted treatment options in patients with non-Hodgkin lymphoma (NHL), preclinical and clinical studies have indicated that in solid cancers only small volume or minimal residual disease might be a suitable target for this treatment modality.[9] In an animal model of PC using Wag/Rij rats with intraperitoneal CC-531 tumors we have shown that the therapeutic effects of CS combined with adjuvant RIT using the ^{177}Lu-labeled MG1 MAb was significantly better than that of CS or RIT alone.[10] In that study RIT was given three days after surgery. We hypothesized that the efficacy of RIT after CS might be dependent on the timing of postoperative administration of the radiolabeled MAbs. To test this hypothesis, we investigated the efficacy of adjuvant RIT when given at various timepoints after CS.

Materials and methods

Animals

Male Wag/Rij-rats, ten to twelve weeks, weighing 240-260 g were obtained from Harlan (Horst, The Netherlands). Rats were accustomed to laboratory conditions for one week prior to experimental use and housed under nonsterile, standard laboratory conditions (temperature, 20–24°C; relative humidity, 50-60%; 12 h light/12 h dark) on sawdust in filter-topped cages (two rats per cage) with free access to animal chow (Snif

Voer, Soest, The Netherlands) and water. All experiments were conducted in accordance with the revised Dutch Act on Animal Experimentation (1997) and approved by the institutional Animal Welfare Committee of the Radboud University Nijmegen.

Cell line

The syngeneic rat colon carcinoma cell line CC-531, derived from colon tumors of Wag/Rij rats exposed to 1,2-dimethyl-hydrazine, was used.[11] CC-531 was cultured and maintained as monolayers on plastic in Dulbecco's Modified Eagle Medium (DMEM, GIBCO, BRL Life Sciences Technologies, The Netherlands) supplemented with 10% fetal calf serum (GIBCO), 2 mM L-glutamine, penicillin (100 units/mL) and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere with 5% CO_2. Prior to inoculation, tumor cells were washed with phosphate-buffered saline (PBS), harvested with 0.25% trypsin (3 min at 37 °C), resuspended in PBS, spun down (5 min at 700 x g), counted and resuspended once again in PBS to the appropriate concentration.

Monoclonal antibody

The MG1 MAb was purchased from Antibodies for Research Applications BV (Gouda, The Netherlands). The MG1 MAb is a murine IgG_{2a} antibody, raised by immunization of mice with CC-531 colon carcinoma cells.[12,13] It recognizes a cell surface structure of about 80 kDa and localizes to tumor cells when injected in rats bearing CC-531 tumors with minimal cross reactivity to other cell types. Purity was checked by SDS-PAGE under nonreduced conditions and by means of fast protein liquid chromatography (FPLC) on a Phenomenex Biosep 3000 column, eluted with phosphate buffered saline (PBS, pH 7.2, 1 mL/min).

^{177}Lu-labeling

^{177}Lu was purchased from IDB Holland BV (Baarle Nassau, The Netherlands). ^{177}Lu-labeling was performed under strict metal-free conditions. To allow labeling of the antibodies with ^{177}Lu, the MG1 MAb was conjugated with isothiocyanatobenzyl-diethylenetriaminepentaacetic acid (ITC-DTPA, Macrocyclics, Dallas, TX). Briefly, ITC-DTPA was conjugated to MG1 in a 0.1 M carbonate buffer, pH 9.0 using a 100-fold molar excess of ITC-DTPA as described by Ruegg et al.[14] with minor modifications (conjugation period of one hour at room temperature). The DTPA-MG1 immunoconjugate was then purified by extensive dialysis against 0.1 M ammonium acetate buffer, pH 5.4. The number of DTPA ligands per antibody molecule was determined according to the method described by Hnatowich et al.[15] The purified DTPA-MG1 conjugate (DTPA/MG1 ratio 1.6 : 1, 1.0 mg/mL) was incubated with ^{177}Lu in 0.1 M ammonium acetate buffer, pH 5.4 at room temperature (60 minutes). Labeling efficiency of the labeling procedure exceeded 98%. Specific activities of the ^{177}Lu-DTPA-MG1 preparation

(hereafter referrend to as ^{177}Lu-MG1) was 0.74 MBq/μg. Since ^{177}Lu is a bone-seeking radionuclide, after completion of the labeling procedure 1 mM EDTA was added to the solutions to scavenge the remaining free ^{177}Lu ions.[16]

Quality control of the radiolabeled preparations

The reaction mixture was purified on a PD-10 column, eluted with PBS, 0.5% BSA. Radiochemical purity (RCP) was determined using instant thin-layer chromatography (ITLC) on silicagel strips (Gelman Sciences, Ann Arbor, MI) using 0.10 M citrate buffer (pH 6.0) as the mobile phase. The RCP of the ^{177}Lu-MG1 preparation was 99%.
The immunoreactivity of the radiolabeled MG1 preparations was essentially determined as described by Lindmo et al.[17] Briefly, a fixed amount of labeled antibody (10,000 cpm) was incubated (6 hours, 37°C) with increasing concentrations of CC-531 tumor cells (1.2×10^6 – 20×10^6 cells/mL) in 0.5 mL binding buffer (RPMI medium containing 0.5% BSA and 0.05% NaN_3). A duplicate of the lowest cell concentration was incubated in the presence of an excess unlabeled antibody to correct for nonspecific binding. After six hours of incubation at 37°C, the cells were spun down (500 g, 5 min) and activity in the pellet was determined in a well-type gamma-counter. The inverse of the tumor cell bound fraction was plotted against the inverse of the cell concentration and the immunoreactive fraction (IRF) was calculated from the y-axis intercept. Immunoreactivity of the ^{177}Lu-MG1 preparation was 67%.

Experimental design

Seven days after intraperitoneal inoculation of 2.0×10^6 CC-531 tumor cells, 75 rats were randomly assigned to undergo exploratory laparotomy (EL) only, CS only, CS + RIT administered immediately postoperatively (CS + RIT 0 group), CS + RIT administered four days postoperatively (CS + RIT 4 group), or CS + RIT administered fourteen days postoperatively (CS + RIT 14 group) (fifteen rats per group). Subsequently, all rats underwent a complete midline laparotomy during which the intraperitoneal tumor load was semiquantitatively scored as described previously.[10] In brief, after opening the abdomen, the abdominal cavity was carefully inspected, starting with the upper abdomen, including the greater omentum, the liver hylum, the perisplenic region and the diaphragm. Subsequently, the gonadal fat pads and the kidneys were inspected. Finally, the intestines were gently lifted out of the abdomen, enabling inspection of the complete mesentery and Douglas' pouch. Tumor growth at each of these sites was scored 0, 1, 2, or 3, where 0 indicated no macroscopic tumor growth, 1 meant little tumor growth, 2 indicated moderate tumor growth, and 3 indicating abundant tumor growth. The sum of the tumor scores of all sites represented the peritoneal cancer index (PCI). RIT consisted of 56 MBq ^{177}Lu-MG1 (total MG1 protein dose 75 μg), which was injected intraperitoneally.

Follow-up

The primary endpoint was survival. Body weight was measured daily in the first week postoperatively and once weekly thereafter as measure of toxicity. Rats were monitored daily until the humane endpoint had been reached, as determined by one single experienced and independent animal technician, who was blinded to the therapeutic regimen. At the time of the humane endpoint, rats were usually lethargic, showing signs of advanced PC, such as the presence of ascites or bulky intraperitoneal tumor growth, and were expected to die within one or two days. When the humane endpoint had been reached, rats were euthanized by O_2/CO_2-asphyxiation and immediately dissected. The experiment was terminated at 118 days after tumor cell inoculation when the remaining rats were euthanized and dissected. In case of absence of macroscopic tumor growth, relevant organs, including the greater omentum, the mesentery and the diaphragm were removed for routine histopathological hemotoxilin & eosin (H&E) and/or immunohistochemical staining using the murine MG1 antibody and a horse-anti-mouse IgG antibody (Vector Laboratories Inc., Burlingame, CA, USA).

Statistical analysis

Data are expressed as means ± standard deviation (SD) unless stated otherwise. Statistical analysis was performed using the SPSS software (Chicago, IL) software and Graphpad Prism 4.00 (Graphpad Software Inc. San Diego USA). Comparison of dichotomous values was done using Chi square or Fisher's Exact test and post-hoc testing with homogeneity of variance correction using Games-Howell. Multiple comparisons were analyzed using the one-way ANOVA test. Bonferroni correction for multiple testing was applied. Survival portions were analyzed using the Log-rank test. All tests were two-sided; the level of statistical significance was set at a P-value of <0.05.

Results

Surgery

All surgical procedures went uneventful, except for one rat that needed resuscitation twice (probably due to too deep anesthesia) and one rat in which the cecum was accidentally perforated while resecting a tumor lesion in the mesentery (without further adverse events). At laparotomy all rats had macroscopic tumor growth, mainly in the greater omentum, the liver hilum, the perisplenic region and the mesentery. In all rats, except those that underwent EL only, an omentectomy was routinely carried out. CS did not require splenectomy in any of the rats. In 53 out of the 60 rats undergoing CS, the resection of all tumor nodules seemed macroscopically complete, whereas in

Table 1. Surgical and pathological characteristics at laparotomy

	EL only	CS only	CS + RIT 0	CS + RIT 4	CS + RIT 14
Tumor score per site†					
Subcutaneously	0 (0-3)	0 (0-1)	0 (0-1)	0 (0-1)	0 (0-1)
Greater omentum	2 (2)	2 (2)	2 (2-3)	2 (2-3)	2 (2-3)
Liver hylum	1 (0-1)	1 (0-1)	1 (0-1)	1 (0-1)	1 (0-1)
Perisplenic	0 (0)	0 (0-1)	0 (0)	0 (0)	0 (0-1)
Mesentery	1 (0-1)	1 (0-3)	1 (0-2)	1 (0-2)	1 (0-2)
Gonadal fatpads	0 (0-1)	0 (0-2)	1 (0-2)	1 (0-1)	1 (0-2)
Diaphragm	0 (0)	0 (0)	0 (0-1)	0 (0-1)	0 (0-1)
Parietal peritoneum	1 (0-1)	1 (0-1)	1 (0-1)	1 (0-2)	1 (0-1)
Total	5 (3-6)	5 (3-7)	5 (3-8)	5 (3-7)	6 (3-7)
Resection macroscopically complete					
Yes	NA	14	14	13	12
No	NA	1	1	2	3

† Values are given as medians (range). EL, exploratory laparotomy; CS, cytoreductive surgery; RIT 0, radioimmunotherapy given immediately postoperatively; RIT 4, radioimmunotherapy given 4 days postoperatively. RIT 14, radioimmunotherapy given 14 days postoperatively

seven rats macroscopic disease had to be left behind, in all cases located in the liver hilum. In those seven rats that had undergone an incomplete cytoreduction, the size of the residual disease was always less than 1 mm. As shown in Table 1, all treatment groups were well balanced with regard to PCI and whether or not the cytoreduction had been complete.

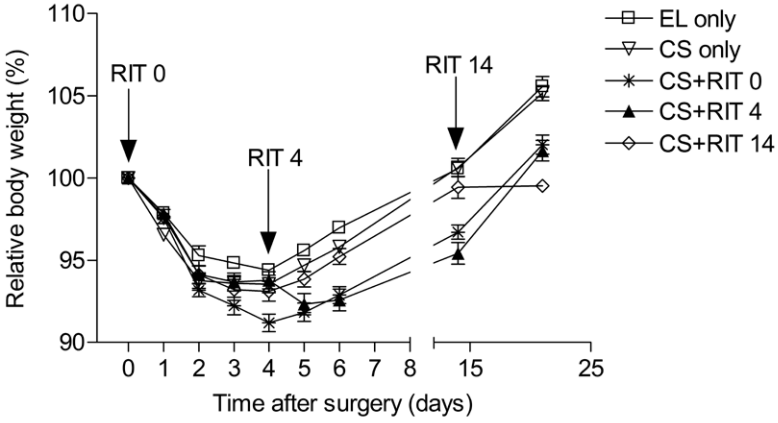

Figure 1
The relative body weight of Wag/Rij rats with small peritoneal CC-531 metastases in the first three weeks after exploratory laparotomy (EL) only, cytoreductive surgery (CS) only, CS + radioimmunotherapy given immediately postoperatively (CS + RIT 0), CS + radioimmunotherapy given four days postoperatively (CS + RIT 4), or CS + radioimmunotherapy given 14 days postoperatively (CS + RIT 14). Data represent means ± standard error of the mean (SEM).

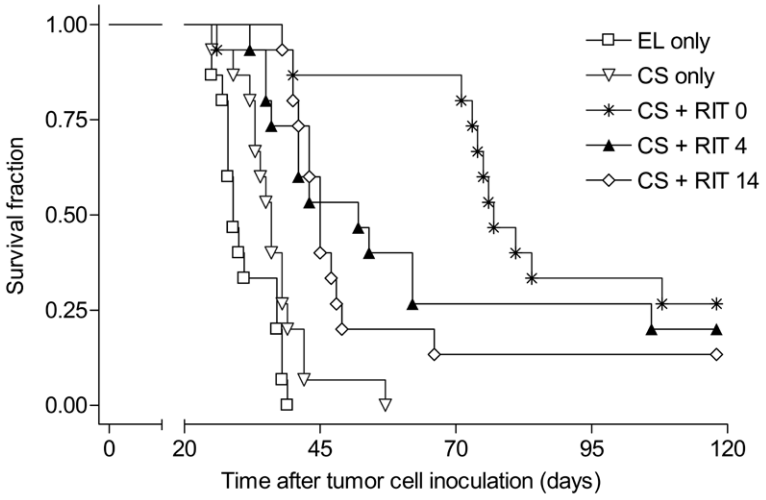

Figure 2
Survival curves for Wag/Rij rats with small peritoneal CC-531 metastases after exploratory laparotomy (EL) only, cytoreductive surgery (CS) only, CS + radioimmunotherapy given immediately postoperatively (CS + RIT 0), CS + radioimmunotherapy given four days postoperatively (CS + RIT 4), or CS + radioimmunotherapy given 14 days postoperatively (CS + RIT 14).

Toxicity

The relative body weight, expressed as the percentage of the body weight on the day of surgery, is depicted in Figure 1. Maximum body weight loss after EL or CS only was similar (5.6 ± 1.4% vs. 6.5 ± 2.0% four days postoperatively, P=0.272). Those rats that were given adjuvant RIT immediately postoperatively had a maximum body weight loss of 8.8 ± 2.1%, which was significantly higher than that after EL (P=0.0001) or CS only (P=0.003). Maximum body weight loss of those rats that received adjuvant RIT four days postoperatively was 7.7 ± 2.5% five days postoperatively. The body weight of those rats that were treated with adjuvant RIT fourteen days postoperatively was not measured in the week following RIT. However, at 21 days after surgery body weight loss of the rats that received RIT fourteen days postoperatively, i.e. 7 days after RIT, was significantly lower as compared to that of the rats that received adjuvant RIT immediately or four days postoperatively (one-way ANOVA test, P=0.004).

Survival

64 Rats died as a result of intraperitoneal tumor growth, in most cased accompanied by the formation of ascites. Mean ascites weight varied from 25.9 ± 18.2 g in the rats that received adjuvant RIT four days postoperatively to 35.1 ± 10.1 g in the rats that underwent EL only (P=0.60). Two rats, one of the CS group and one of the CS + RIT 0 group, were euthanized because of massive weight loss at 25 days and 26 days after tumor cell inoculation, respectively. At dissection these rats had only minor tumor growth, that could not explain their deteriorating condition. The survival curves, including all rats, are shown in Figure 2. Median survival of animals in the rats that underwent EL or CS only was 29 days (range 25-39) and 39 days (range 25-57), respectively (P=0.039). The rats that received adjuvant RIT immediately postoperatively, or four or fourteen days postoperatively was 77 days (range 26-118), 52 days (range 32-118) and 45 days (range 33-118), respectively. Adjuvant RIT significantly improved survival relative to CS only, at all time points administered (P<0.002 for all comparisons), with a highy significant trend towards improved survival with earlier administration (P<0.0001). The median survival after adjuvant RIT given immediately postoperatively was significantly better when compared to that after RIT administered fourteen days postoperatively (P=0.02). The difference between the survival of the rats that were given RIT immediately postoperatively and that of the rats that received RIT four days postoperatively, however, was not significant (P=0.17).

Long-term survivors

118 Days after tumor cell inoculation nine rats (four of the CS + RIT 0 group, three of the CS + RIT 4 group and two of the CS + RIT 14 group) were still alive, without clinical evidence of PC. At dissection, one rat of the CS + RIT 0 group, two rats of

the CS + RIT 4 group and both rats of the CS + RIT 14 group had macroscopic tumor growth. In those rats without macroscopic evidence of residual disease, histopathological examination of relevant organs including the diaphragm, the omental remnant, and the mesentery revealed minimal residual disease in one rat of the CS + RIT 0 group.

Discussion

The aim of the present experimental study was to compare the efficacy of adjuvant RIT when given at various time points after CS of PC of colonic origin. Although adjuvant RIT improved survival relative to CS alone at every time point tested, there was a highly significant trend towards improved survival with earlier administration of the radiolabeled antibodies.

The present animal model of PC using Wag/Rij rats and the syngeneic colon carcinoma cell line CC-531 was used because of the reproducible tumor growth in these rats and its similarity to the clinical presentation of PC.[18] The MG1 MAb has previously been shown to localize preferentially in CC-531 liver tumors in Wag/Rij rats.[12,19] In a biodistribution study in rats with intraperitoneal CC-531 tumor lesions the uptake of ^{125}I-labeled MG1 was lower than that of ^{111}In-labeled MG1 (1.1% ID/g vs 4.1% ID/g), which is probably due to the different handling of the radiolabeled catabolites of the antibody after internalization of the radiolabeled antibody by the tumor cells.[10] In view of the higher uptake of ^{111}In-MG1 we decided to use ^{177}Lu in RIT studies. The beta-emitter ^{177}Lu has several favorable physical characteristics for RIT, including a half-life of 6.7 days and a medium-energy beta-emission, resulting in a maximum penetration range in tissue of 2.5 mm. ^{177}Lu is therefore considered a very suitable radionuclide for RIT in small volume disease, as demonstrated in preclinical studies.[20]

In a mouse model of PC of colonic origin, we previously demonstrated that intraperitoneal administration of radioiodinated anti-CEA MAbs results in higher uptake in small (1-3 mm) tumor lesions than intravenous administration up to 72 hours postinjection.[20,21] Therefore, in the present study RIT was given intraperitoneally.

The marked differences in survival between those rats that received adjuvant RIT immediately postoperatively and the rats that received RIT four of fourteen days later might be related to several factors. Firstly, abdominal surgery inevitably results in peritoneal trauma, which elicits an inflammatory response and the production of fibrinogen-rich peritoneal exudate.[22] Activation of the coagulation cascade subsequently results in the production of thrombin, which catalyzes the conversion of fibrinogen into fibrin. It has been hypothesized that tumor cells can be encapsulated in the fibrin network and as such be less accessible to local therapy, such as chemotherapy or antibodies (tumor cell entrapment theory).[23] In the present study the formation of fibrin might have hampered tumor targeting of the radiolabeled MG1 antibodies and consequently might have impaired their therapeutic efficacy at four and fourteen days

postoperatively. Secondly, the production of fibrin is a common pathway for the development of adhesions, which are inevitably formed after abdominal surgery.[24] Intra-abdominal adhesions may have hampered the distribution of the radiolabeled MAbs over the peritoneal surfaces in the rats that received adjuvant RIT four or fourteen days postoperatively. Thirdly, since CC-531 is a rapidly growing cell line, microscopic residual disease might have grown to macroscopic disease, especially in the rats that received adjuvant RIT fourteen days postoperatively. The uptake and consequently therapeutic efficacy of radiolabeled antibodies is inversely correlated with tumor size.[9] The growth of minimal residual disease into larger tumors might therefore have had a negative effect on therapeutic efficacy of the radiolabeled MAbs.

To date two clinical trials have been published investigating the efficacy of adjuvant RIT for the treatment of minimal residual disease, one of which in patients with PC. In a phase II trial Liersch et al. investigated the safety and efficacy of adjuvant RIT using the ^{131}I-labeled humanized anti-CEA MAb Labetuzumab (MN-14) in 23 patients who had undergone Ro liver resection for metastatic colorectal cancer.[25] The major side effect was transient grade 3 or less neutropenia and/or thrombopenia. Median disease-free and overall survival was 18 months (95% CI 11-31) and 68 months (95% CI 41-infinity), with a five-year survival rate of 51%. The authors concluded that since these results seemed to be improved when compared to historical and contemporaneous controls, a phase III randomized controlled trial is justified.

In a phase III trial Verheijen et al. compared the efficacy of a single intraperitoneal administration of the ^{90}Y-labeled murine anti-MUC1 HMFG1 MAb plus standard treatment to standard treatment alone in patients with stage Ic to IV ovarian cancer.[26] Patients were randomized after they had attained a laparoscopically confirmed complete remission after CS and platinum-based chemotherapy. The radiolabeled antibodies were administered intraperitoneally via a CAPD catheter, after scintigraphic confirmation of equal intra-abdominal distribution using a tracer dose of ^{111}In-labeled HMFG1. RIT using ^{90}Y-HMFG1, however, did not prolong disease-free nor overall survival. Several comments on this RCT seem justified. Firstly, the selection of the high-energy beta-emitter ^{90}Y with a maximum tissue penetration of 12 mm does not seem appropriate, since most of the energy will have been deposited outside the microscopic tumor nodules. Secondly, the radiolabeled antibody preparation was augmented with 20 mg of unlabeled antibody to a total of 25 mg ^{90}Y-HMFG1, with the intent to provoke a human-anti-mouse-antibody response. However, the high antibody dose might have had a negative effect on the uptake of the radiolabel in the tumor lesions.[21] Thirdly, in view of the results of the present study, the time interval between CS and the administration of at least two months might have had a negative impact on tumor targeting.

In conclusion, the results of the present experimental study confirm the potential therapeutic efficacy of RIT when given as adjuvant treatment after CS in PC of colonic origin. Timing of RIT greatly affected the efficacy of the radiolabeled antibodies. In

clinical trials studying the efficacy of radiolabeled antibodies after CS in patients with PC, the adjuvant RIT should therefore be administered as early as possible.

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General discussion & future prospects

The experimental studies described in the present thesis showed that radioimmunotherapy can be an effective treatment modality in small volume experimental peritoneal carcinomatosis of colorectal origin. In nude mice with small peritoneal metastases of the human colon carcinoma cell line LS174T, radioimmunotherapy using the radiolabeled high-affinity murine anti-CEA MAb MN-14 effectively delayed tumor growth. The efficacy of radioimmunotherapy, however, could not be enhanced by combining radioimmunotherapy with either gemcitabine or the selective COX-2 inhibitor parecoxib. Still, in a rat model using the syngeneic rat colon carcinoma cell line CC-531 and the radiolabeled murine anti-CC-531 MAb MG1 the combination of cytoreductive surgery and adjuvant radioimmunotherapy had an effect that was at least additive. These results provided proof of principle that radiolabeled MAbs can constitute an effective adjuvant treatment modality after resection of tumors with a high risk of recurrence.

Whereas 5-fluorouracil (5-FU) has been the only cytostatic agent available for chemotherapy in patients with colorectal cancer for forty years, two new cytostatic agents, irinotecan and oxaliplatin, have been introduced in the new millennium.[1] In addition, targeted therapies using the anti-vascular endothelial growth factor MAb bevacizumab and the anti-epidermal growth factor receptor MAb cetuximab have been added to the therapeutic armamentarium.[2] These changes represent important progress, both for patients requiring adjuvant therapies after surgery as well as for patients requiring medical therapy for recurrent disease. To date, there is little data with regard to the efficacy of these agents in patients with peritoneal carcinomatosis of colorectal origin.[3] In the nude mouse model used in the experimental studies described in the present thesis, the efficacy of radioimmunotherapy should therefore preferably be compared with that of 5-FU, irinotecan and oxaliplatin. Furthermore, 5-FU, irinotecan and oxaliplatin are well-documented radiosensizers, that could potentially enhance the efficacy of radioimmunotherapy. Thus, the efficacy of radioimmunotherapy in the present mouse model should be compared to that of modern medical therapy and be further optimized using radiosensitizers.

The experimental study on the efficacy of radioimmunotherapy after cytoreductive surgery in rats with peritoneal carcinomatosis described in Chapter 7 has been the first investigating the efficacy of adjuvant radioimmunotherapy after resection of tumors with a high risk of recurrence. This animal model could be further optimized with regard to the maximal tolerated activity dose that can be administered safely. More importantly, the efficacy of radioimmunotherapy should be compared to that of (hyperthermic) intraperitoneal chemotherapy, as currently given after cytoreductive surgery in patients with peritoneal carcinomatosis of colorectal origin. As described in Chapter 2, in patients undergoing cytoreductive surgery followed by intraperitoneal chemotherapy, Mitomycin-C (MMC) was the cytostatic agent frequently used. In this regard, Pelz et al. recently published the results of an experimental study of (hyperthermic) intraperitoneal chemotherapy using MMC in a similar rat model of peritoneal carcinomatosis, using Wag/Rij rats and CC-531 tumors.[4] In that study rats were subjected to either hyperthermic peritoneal perfusion with MMC at 15 mg/m^2 or perfusion with MMC at 10 mg/m^2 only (no hyperthermia) using an open-abdomen technique. Control rats received no treatment.

The maximum weight loss in the treatment groups was only 5%, whereas hyperthermic intraperitoneal chemotherapy resulted in a significant reduction of intraperitoneal tumor growth ten days after treatment. After adding cytoreductive surgery to hyperthermic intraperitoneal chemotherapy in this animal model, cytoreductive surgery followed by hyperthermic intraperitoneal chemotherapy could elegantly be compared to cytoreductive surgery followed by adjuvant radioimmunotherapy.

The experimental studies described in the present thesis were performed in mice or rats. Hence, although apparently promising, it is too early to draw conclusions on the potential of radioimmunotherapy for the treatment of peritoneal carcinomatosis in patients. Before trying to answer the question of how to introduce this rather sophisticated treatment modality in the complex surgical treatment protocol currently inflicted on patients with peritoneal carcinomatosis, valid arguments for its necessity need to be identified.

Although the results of the patient series published to date indicate that the outcome of patients undergoing cytoreductive surgery can probably only be improved by means of more effective adjuvant treatment strategies, there is at present no evidence for or against the efficacy of hyperthermic intraperitoneal chemotherapy.[3] The only way to ascertain the efficacy of hyperthermic intraperitoneal chemotherapy after cytoreductive surgery is to compare cytoreductive surgery followed by hyperthermic intraperitoneal chemotherapy to cytoreductive surgery alone. Indeed, one such trial has been conducted.[5] Unfortunately, the trial had to be stopped prematurely after inclusion of only 35 patients due to difficulties in patient recruitment. Still, two- and five-year survival rates of both treatment arms were very similar (60% vs 55% and 20% vs 25%, respectively). Several experts in the field argue that only surgery-related factors have been linked to treatment success and question the efficacy of hyperthermic intraperitoneal chemotherapy, pleading for a new trial similar to that conducted by Elias et al.[6,7] The doubts concerning the efficacy of hyperthermic intraperitoneal chemotherapy also give room for the clinical trials investigating the efficacy of other adjuvant treatments, such as radioimmunotherapy.

Clinical evidence favoring the application of radioimmunotherapy for the treatment of peritoneal metastases is very limited and predominantly stems from clinical series of patients with ovarian cancer (see Chapter 1). Recently, Verheijen et al. published the results of an open-label randomized trial investigating the efficacy of adjuvant radioimmunotherapy using ^{90}Y-labeled murine anti-MUC1 HMFG-1 MAb after cytoreductive surgery and standard consolidation chemotherapy in patients with ovarian cancer.[8] After a laparoscopically confirmed complete remission, patients were randomly assigned to receive to either ^{90}Y-HMFG1 (666 MBq/m^2, one single administration) or no treatment. Aiming at both an antitumor response due to selective uptake of the radionuclide as well as due to a human-antimurine-antibody (HAMA) response, each patient of the radioimmunotherapy arm received a HMFG1 MAb protein dose of 25 mg. Adjuvant radioimmunotherapy, however, failed to improve overall or disease-free survival. Several comments on this randomized trial seem justified. Firstly, the choice for the high-energy beta-emitter ^{90}Y for radioimmunotherapeutic purposes for the treatment of minimal residual disease is questionable. Given the rather high tissue penetration range in tissue (up to 12 mm), most

of the radiation energy has been deposited outside the tumor lesions that were targeted. Secondly, the very high MAb protein dose of 25 mg per patient might have had a negative impact on the uptake of activity in the tumor lesions. The only conclusion that can therefore be reliably drawn from this randomized trial is that the murine HMFG1 MAb has no measurable antitumor effect in patients with minimal residual ovarian cancer.
Based on the results of the experimental radioimmunotherapy as described in the present thesis, one might hypothesize that radioimmunotherapy could be a valuable adjuvant treatment modality after cytoreductive surgery in patients with peritoneal carcinomatosis of colorectal origin, provided that the antibody protein dose and the radionuclide are selected with care. The previously mentioned randomized trial comparing cytoreductive surgery followed by hyperthermic intraperitoneal chemotherapy to cytoreductive surgery alone could therefore be completed by adding a third treatment arm, consisting of cytoreductive surgery followed by radioimmunotherapy. Given the promising results of adjuvant radioimmunotherapy using the radioiodinated humanized (h)MN-14 MAb after resection of liver metastases in patients with colorectal cancer, ^{131}I-hMN-14 or ^{177}Lu-hMN-14 seem to be suitable radionuclide-MAb constructs.[9]

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Summary

The working hypothesis for the experimental work described in this thesis was established in view of the clinical picture of a small group of patients with peritoneal carcinomatosis of colorectal origin, for whom there still is no effective treatment. Although aggressive cytoreductive surgery, resecting as much macroscopic disease as is considered feasible, followed by hyperthermic intraperitoneal chemotherapy has been shown to be effective in selected patients with peritoneal carcinomatosis of colorectal origin, most patients eventually present with intraperitoneal recurrence and succumb to their disease. Since most patients with favorable clinicopathological characteristics, i.e. those in whom a macroscopically complete resection is achieved, die from their disease, one might conclude that the chemotherapy given is not sufficiently effective and that other therapies could be of benefit. The success of radioimmunotherapy using radiolabeled monoclonal antibodies (MAbs) directed against tumor-associated antigens for the treatment of non-Hodgkin lymphoma (NHL) inspired us to hypothesize that this treatment modality could be used in peritoneal carcinomatosis of colorectal origin. The experimental studies described in het present thesis therefore aimed to investigate the therapeutic potential of radioimmunotherapy in small volume and resectable peritoneal carcinomatosis of colorectal origin.

After a short historical overview of radioimmunotherapy, **Chapter 1** provides a detailed description of various relevant technical aspects of radioimmunotherapy, and finally, an extensive review of the clinical results of radioimmunotherapy in various malignancies, including NHL, breast cancer, renal cell cancer, ovarian cancer, and colorectal cancer. While radioimmunotherapy has become one of the standard treatment options for patients with NHL, inefficient localization of radiolabeled MAbs to nonhematological cancers due to various tumor-related factors has refrained radioimmunotherapy from outgrowing the experimental stage in solid tumors. Still, small volume or minimal residual disease has been recognized as a potentially suitable target for radiolabeled antibodies. Focusing on patients with colorectal cancer, twenty-three phase I/II studies have been published investigating the feasibility and efficacy of radioimmunotherapy using five radionuclides and fifteen MAbs against carcinoembryonic antigen (CEA), tumor-associated glycoprotein (TAG)-72, Epithelial cellular adhesion molecule (Ep-CAM), A33 or Colon specific antign (CSA)-p mainly in patients with advanced colorectal cancer. A few responses were recorded. Given the few responses observed in patients with bulky disease and the fact that uptake of radiolabeled MAbs in tumors is inversely related to tumor size, radioimmunotherapy still might be an effective treatment modality for patients with minimal residual disease, small volume disease or as adjuvant treatment. It was concluded that future studies should be focused on its application in patients with small volume disease, e.g. as adjuvant treatment modality after tumors with a high risk of recurrence.

Although hematogenous dissemination to the liver and lungs still poses the greatest threat to patients with colorectal cancer, peritoneal carcinomatosis can be a major source of morbity and is associated with a poor prognosis. **Chapter 2** describes the incidence of peritoneal seeding of tumor cells during surgical resection of colorectal cancer, which presumably is an important pathophysiological mechanism of intraperitoneal dissemination. Furthermore, the incidence rates of intraperitoneal recurrence as well as the current treatment strategies for peritoneal carcinomatosis are discussed. Peritoneal seeding of cancer cells proved to be a relatively common event during surgery for colorectal cancer (3%-28% during curative surgery) although its clinical significance remains obscure. Furthermore, macroscopically evident peritoneal carcinomatosis at primary diagnosis (up to 7%) and local recurrence or peritoneal carcinomatosis after potentially curative surgery (4%-36%) is encountered more frequently than often realized. The small number of four patient series dedicated to the efficacy of chemotherapy for the treatment of peritoneal carcinomatosis of colorectal origin, once more indicates that this site of tumor recurrence has for long been regarded as less important than the liver. Treatment with palliative systemic 5-FU based chemotherapy seems less efficacious than aggressive cytoreductive surgery followed by intraperitoneal chemotherapy aimed at locoregional control of disease. Still, the role of modern cytostatic agents, such as irinotecan or oxaliplatin, remains to be elucidated.

Although the clinical results of radiolabeled MAbs in the treatment of various solid cancers, including colorectal cancer, have been modest, preclinical research has indicated that radioimmunotherapy can effectively inhibit the growth of small lesions, and in some instances even ablate them. In **Chapter 3** the mouse model used in the preclinical studies of this thesis is described and characterized. Nude mice were used, in which peritoneal carcinomatosis was induced by intraperitoneal inoculation of the human colon carcinoma cell line LS174T. The macroscopic appearance of the intraperitoneal tumor xenografts resembles the clinical picture of peritoneal carcinomatosis seen in man, including preferential tumor growth in the greater omentum, the liver hylum and on the diaphragm and, eventually, the formation of hemorrhagic ascites. Since LS174T tumor cells, like most human colon carcinoma cells, express the carcinoembryonic antigen (CEA), the murine high-affinity anti-CEA MAb MN-14, developed at the Center for Molecular Medicine and Immunology (CMMI, Belleville, New Jersey, USA) was used. This antibody has already been successfully applied for radioimmunotherapy in clinical trials. Before initiating therapy studies, first the effect of the MN-14 protein dose on the uptake in tumor tissue and the optimal route of administration (intraperitoneal vs. intravenous) was investigated. In a protein dose-escalation study, it was demonstrated that the uptake in tumor and tumor-to-blood ratio significantly decreased when the MN-14 protein dose exceeded 25 μg and 20 μg respectively, indicating saturation of the CEA epitopes in the tumor nodules. Intraperitoneal adminstration, furthermore, resulted in a higher uptake in tumor and higher tumor-to-blood ratios up to 48 hours post-injection (p.i.). Indeed, dosimetric analysis of the

biodistribution data (taking the physical half-life, energy and tissue penetration of the emitted electrons into account) indicated that intraperitoneal administration would consequently result in a 25% higher tumor absorbed radiation dose than intravenous administration. These data favored intraperitoneal over intravenous administration of no more than 20 μg MN-14, labeled with therapeutic activity doses. Finally, in the therapy studies, intraperitoneal radioimmunotherapy using ^{131}I-labeled MN-14 proved to be highly effective, even at relatively low activity doses.

Although ^{131}I is well-known, cheap, easy to label, and therefore probably the most frequently used radionuclide used in radioimmunotherapy, several other radionuclides, such as ^{186}Re, ^{90}Y, and ^{177}Lu are available that also have suitable characteristics for application in radioimmunotherapy. In **Chapter 4** the results of experimental biodistribution as well as therapy studies are reported, that aimed to select of the most effective radionuclide in the present model. For this purpose, the biodistribution of MN-14 labeled with either ^{186}Re or ^{88}Y (as surrogate marker for ^{90}Y and ^{177}Lu) was assessed, both after intraperitoneal and intravenous administration and compared with that of radioiodinated MN-14. Again, uptake of ^{186}Re-labeled or ^{88}Y-labeled MN-14 in tumor was higher after intraperitoneal adminitration than after intravenous administration. Furthermore, uptake of ^{88}Y-labeled MN-14 in tumor was higher than that of ^{186}Re-labeled or radioiodinated MN-14, which probably ensues from differences in the fate of the radionuclides after intratumoral catabolization of the radiolabeled antibodies, favoring the use of MN-14 labeled with either ^{90}Y or ^{177}Lu. Dosimetric analysis indicated that radioimmunotherapy using MN-14 labeled with ^{177}Lu or ^{131}I resulted in the highest tumor absorbed radiation doses. Indeed, the survival of mice treated with ^{177}Lu-labeled or ^{131}I-labeled MN-14 displayed the highest median survival as compared to the mice treated with either ^{90}Y-labeled or ^{186}Re-labeled MN-14. Based on the results of the biodistribution and therapy studies, it was concluded that ^{177}Lu and ^{131}I were the most suitable radionuclides to be used for radioimmunotherapy of small volume peritoneal carcinomatosis.

After having characterized and optimized the experimental model, strategies were pursued to further improve the efficacy of radioimmunotherapy in the present model. Since there is increasing evidence that inhibition of the cyclo-oxygenase (COX)-2 enzyme can sensitize tumor cells to external beam radiation therapy, we hypothesized that COX-2 inhibition might also sensitize the COX-2 positive LS174T tumors to radioimmunotherapy. In **Chapter 5** the results of experimental studies are described that aimed to test this hypothesis. For this purpose parecoxib, a analgesic drug developed for parenteral administration, was used. Parecoxib is a prodrug that after parenteral administration is converted to the active agent valdecoxib, which is the strongest COX-2 inhibitor currrently available on the market. Parecoxib monotherapy consisting of fourteen daily intraperitoneal administrations at 0.2 – 25 mg/kg had no measurable antitumor effect, nor did parecoxib co-administration affect the biodistri-

bution of radiolabeled MN-14. When administered in combination with radioimmunotherapy, parecoxib failed to enhance the efficacy of radioimmunotherapy.

In **Chapter 6** a similar attempt was made to sensitize the LS174T tumors to radiation by co-administration of gemcitabine, a cytostatic agent with strong radiosensitizing potential. The choice for gemcitabine was furthermore inspired by previous reports, demonstrating that gemcitabine could indeed enhance the efficacy of radioimmunotherapy in animal models of pancreatic and colon cancer. After establishing the maximum tolerated dose that can be administered safely, two administration regimens were tested. The first regimen consisted of four intraperitoneal administrations at 0.11 mg or 0.33 mg every third day, starting on the day of radioimmunotherapy. In the second regimen gemcitabine was administered at 0.022 mg on five consecutive days, again starting on the day of radioimmunotherapy. The maximal tolerated dose of gemcitabine in both treatment regimens was far lower than that reported by other authors, which may be due to differences between mouse strains. Gemcitabine, however, failed to significantly improve the efficacy of radioimmunotherapy in both regimens tested.

In **Chapter 7**, radioimmunotherapy is combined with cytoreductive surgery. Since cytoreductive surgery was considered not feasible in nude mice, a rat model was used. Peritoneal carcinomatosis was induced in Wag/Rij rats by intraperitoneal inoculation of the syngeneic rat colon carcinoma cell line CC-531. The murine MG1 MAb, specifically reactive with CC-531 tumor cells with limited cross-reactivity with normal tissues in Wag/Rrij rats was used. The results of biodistribution studies of MG1 labeled with either ^{125}I or ^{111}In (as surrogate radionuclides for ^{131}I and ^{177}Lu, respectively) indicated that the uptake of ^{111}In-labeled MG1 in the tumor was higher than that of ^{125}I-labeled MG1. Subsequently, the therapeutic efficacy of MG1 labeled with ^{177}Lu was assessed and compared with that of unlabeled MG1 or the carrier. Radioimmunotherapy using ^{177}Lu-labeled MG1 (74 MBq= 2 mCi per rat) nearly eradicated tumor growth, whereas unlabeled MG1 had no antitumor effect at all. Finally, in the surgery study, rats with resectable intraperitoneal CC-531 tumors were subjected to exploratory laparotomy only, cytoreductive surgery only, exploratory laparotomy + radiommunotherapy (55 MBq = 1.5 mCi per rat), or cytoreductive surgery + radiommunotherapy. Whereas cytoreductive surgery monotherapy resulted in a small survival benefit of 10 days, exploratory laparotomy + radioimmunotherapy produced a significant survival benefit of 21 days. The combination of both treatment modalities, however, resulted in a survival benefit of 47 days, which suggests an additive effect of the combination of both treatment modalities. These results provided proof of principle that radioimmunotherapy can be an effective treatment modality, when administered as adjuvant treatment modality after resection of tumors with a high risk of recurrence.

In **Chapter 8**, the hypothesis that the efficacy of adjuvant RIT after cytoreductive surgery depends on the timing of administration, is tested. For this purpose, Wag/Rij rats with resectable peritoneal CC-531 carcinomatosis were subjected to exploratory laparotomy only, cytoreductive surgery only, cytoreductive surgery + radioimmunotherapy administered immediately postoperatively, cytoreductive surgery + radioimmunotherapy administered four days postoperatively, or cytoreductive surgery + radioimmunotherapy administered fourteen days postoperatively. Cytoreductive surgery resulted in a small survival benefit as compared to exploratory laparotomy only. Adjuvant RIT significantly improved survival relative to CS only, at all time points administered ($P<0.002$ for all comparisons), with a highy significant trend towards improved survival with earlier administration. It was concluded that adjuvant radioimmunotherapy after cytoreductive surgery of peritoneal carcinomatosis should be administered as soon as possible.

In **Chapter 9**, the experimental results are discussed and future perspectives are outlined.

Samenvatting

Kwaadaardige tumoren van de dikkedarm (coloncarcinoom) of endeldarm (rectumcarcinoom) zaaien dikwijls uit naar de lymfeklieren, de lever, de longen en het buikvlies (peritoneum). Uitzaaiingen op het buikvlies (peritoneale metastasen) kunnen leiden tot darmobstructie, fistelvorming en ascites. Dit ziektebeeld wordt peritoneale carcinomatose of carcinosis peritonei genoemd. Sommige patiënten hebben alleen peritoneale carcinomatose, zonder metastasen in lever of longen of elders in het lichaam. Tot voor kort werden deze patiënten alleen behandeld met palliatieve chemotherapie en was de gemiddelde overleving ongeveer een jaar. Sinds enkele jaren kunnen deze patiënten operatief worden behandeld. Daarbij worden de in de buikholte aanwezige tumoren zo veel mogelijk verwijderd (chirurgische cytoreductie of 'debulking') waarna de buikholte onder verwarming wordt gespoeld met chemotherapeutica (Hypertherme Intraperitoneale Chemotherapie (HIPEC)). Deze behandeling gaat gepaard met aanzienlijke morbiditeit (ca. 35%) en mortaliteit (ca. 5-10%). Toch lijkt deze agressieve benadering te leiden tot een betere overleving dan gebruikelijke behandeling met alleen chemotherapie. Analyse van de resultaten van deze ingrijpende ingreep heeft ons inmiddels geleerd dat de resultaten mogelijk door effectievere adjuvante behandelingen kunnen worden verbeterd.
Bij radioimmunotherapie wordt gebruik gemaakt van een radionuclide dat aan een antistof (antilichaam) is gekoppeld. Dit antilichaam is gericht tegen eiwitstructuren op tumorcellen (antigenen), zodat het zich na toediening in de buikholte of de bloedbaan kan ophopen in tumoren. Op die wijze kunnen tumorcellen preferentieel worden bestraald, terwijl gezonde weefsels relatief gespaard blijven. Radioimmunotherapie is effectief gebleken in de behandeling van patiënten met lymfeklierkanker (non-Hodgkin lymfoom). Bij de zogenaamde solide tumoren, zoals colon- en rectumcarcinoom, lijkt radioimmunotherapie alleen effectief als de tumor klein is, zoals het geval is in de vroege stadia van peritoneale metastasering of na cytoreductieve chirurgie van peritoneale carcinomatose.
Het doel van de in dit proefschrift beschreven studies was te onderzoeken of radioimmunotherapie werkzaam kan zijn tegen kleine peritoneale metastasen en of radioimmunotherapie mogelijk een waardevolle aanvullende behandeling kan zijn na cytoreductieve chirurgie van peritoneale carcinomatose van colorectale origine.

Na een kort historisch overzicht van de toepassing van radioimmunotherapie, worden in **hoofdstuk 1** verschillende technische aspecten van radionucliden en antilichamen besproken. Bovendien wordt een uitgebreid overzicht gegeven van de klinische resultaten van radioimmunotherapie bij patiënten met lymfeklierkanker, mammacarcinoom (borstkanker), niercelcarcinoom, ovariumcarcinoom (eierstokkanker) en colon- of rectumcarcinoom. Het effect van radioimmunotherapie bij patiënten met lymfeklierkanker is zo goed, dat deze behandeling inmiddels is geregistreerd als een van de standaardbehandelingen voor deze ziekte. De effectiviteit bij de andere genoemde vormen van kanker (de solide tumoren) is echter beperkt. Dit wordt ondermeer veroorzaakt door verschillende tumor-gerelateerde factoren, die de opname van de gelabelde

antilichamen, en dus de hoeveelheid radioactiviteit, in deze tumoren beperken. Wel is gebleken dat de opname van de antilichamen in tumoren omgekeerd evenredig is met de grootte van de tumoren. In geval van kleine tumoren, of minimaal residuale ziekte, zou radioimmunotherapie dus wel degelijk effectief kunnen zijn.
Tot op heden zijn 23 klinische onderzoeken gepubliceerd, waarin patiënten met colon- of rectumcarcinoom werden behandeld met radioimmunotherapie. In totaal werden in deze onderzoeken vijftien verschillende antilichamen gericht tegen hetzij carcinoembryonic antigeen (CEA), tumor-geassocieerd glycoproteine (TAG)-72, epitheliaal cellulair adhesie molecuul (Ep-CAM), A33 of het colon-specifiek antigeen (CSA)-p, en vijf verschillende radionucliden gebruikt. In alle onderzoeken, op één na, werden patiënten geïncludeerd met grote tumoren. Het is daarom opvallend dat toch enkele patiënten een respons vertoonden. Omdat verwacht mag worden dat het effect van radioimmunotherapie groter is in kleine tumoren, werd geconcludeerd dat in toekomstige studies radioimmunotherapie moet worden gegeven in patiënten met kleine tumoren of minimaal residuale ziekte.

In **hoofdstuk 2** wordt nader ingegaan op het pathofysiologisch mechanisme van peritoneale metastasering, de incidentie en de huidige behandelingsmethoden van peritoneale metastasen in patiënten met colon- of rectumcarcinoom. Peritoneale metastasen worden waarschijnlijk veroorzaakt door tumorcellen die zich hetzij reeds voor de operatieve verwijdering in de buikholte bevinden, hetzij tijdens de operatie als gevolg van doorsnijding van bloed- en/of lymfevaten de vrije buikholte bereiken. Het blijkt dat deze vorm van uitzaaien, in het Engels aangeduid met 'peritoneal seeding', kan worden aangetoond in 3-28% van patiënten die met curatieve intentie voor het eerst worden geopereerd in verband met een colon- of rectumcarcinoom. De klinische significantie van het aantreffen van deze cellen is echter nog niet duidelijk. Macroscopisch evidente peritoneale carcinomatose wordt verder gediagnosticeerd in ongeveer 7% van de patiënten ten tijde van de eerste operatie. Na chirurgische verwijdering van de primaire tumor, treedt lokaal recidief of peritoneale carcinomatose op in 4-36% van de patiënten tijdens follow-up. Tot op heden zijn slechts vier onderzoeken gepubliceerd waarin het effect van palliatieve chemotherapie voor de behandeling van patiënten met peritoneale carcinomatose wordt beschreven. Dit onderstreept nogmaals dat uitzaaiingen naar het buikvlies lange tijd zijn gezien als minder belangrijk dan leveruitzaaiingen. Palliatieve chemotherapie op basis van 5-fluorouracil en operatief ingrijpen wanneer noodzakelijk is minder effectief dan agressieve chirurgische cytoreductie gevolgd door intraperitoneale chemotherapie, veelal met mitomycine-C. De plaats van nieuwe cytostatica, zoals irinotecan of oxaliplatin, moet echter nog worden onderzocht.

In **hoofdstuk 3** wordt een muizenmodel beschreven en gekarakteriseerd, waarin de effectiviteit van radioimmunotherapie voor de behandeling van peritoneale carcinomatose wordt onderzocht. Hiervoor werden zogenaamde naakte muizen gebruikt, waarin peritoneale carcinomatose werd geïnduceerd door middel van een injectie met tumor-

cellen van de humane coloncarcinoom-cellijn LS174T in de buikholte. Dit leidt tot een macroscopisch beeld van tumorgroei in de buikholte hetgeen sterk lijkt op dat van peritoneale carcinomatose in de mens. Tumorgroei wordt hierbij met name gezien ter plaatse van het omentum, nabij de lever en op het diafragma (middenrif). Uiteindelijk wordt bloederige ascites gevormd. Omdat LS174T CEA tot expressie brengt, werd het MN-14 antilichaam met hoge affiniteit voor CEA gebruikt. Dit antilichaam is ontwikkeld in het Center for Molecular Medicine and Immunology (CMMI) te Belleville, New Jersey (Verenigde Staten) en reeds succesvol toegepast in patiënten. Allereerst werd onderzocht wat het effect was het de MN-14 eiwit dosis op de opname van MN-14 in de tumoren en via welke route het radioactief gelabeld MN-14 moest worden toegediend om een zo hoog mogelijke opname in de tumoren te bewerkstelligen. Het bleek dat bij een MN-14 eiwit dosis van meer dan 20 µg of 25 µg de tumor/bloed-ratio respectievelijk opname in tumor significant afnamen. Dit wordt waarschijnlijk verklaard door verzadiging van het tumor-geassocieerd CEA. Intraperitoneale toediening leidde voorts tot een hogere opname in tumorweefsel en hogere tumor/bloed ratio's dan intraveneuze toediening gedurende de eerste 48 uur na toediening. Op basis van dosimetrische analyse van de biodistributie-data kon worden berekend dat intraperitoneale radioimmunotherapie zou leiden tot een stralingsdosis op de intraperitoneale tumoren welke 25% hoger lag dan die na intraveneuze radioimmunotherapie. Radioimmunotherapie met ^{131}I-gelabeld MN-14 was inderdaad zeer effectief en vertraagde de tumorgroei zelfs bij relatief lage doses.

^{131}I is goedkoop, gemakkelijk te labelen en het meest gebruikte radionuclide in radioimmunotherapie. Inmiddels is er een aantal nieuwe radionucliden, zoals ^{186}Re, ^{90}Y en ^{177}Lu beschikbaar, die geschikt zijn voor radioimmunotherapeutische toepassing. In **hoofdstuk 4** worden de resultaten beschreven van een aantal experimentele studies, die tot doel hadden het meest geschikte radionuclide te identificeren voor radioimmunotherapie voor kleine peritoneale metastasen. Hiertoe werd de biodistributie bepaald van MN-14 gelabeld met ^{186}Re of ^{88}Y (als surrogaat radionuclide voor ^{90}Y en ^{177}Lu), zowel na intraveneuze als na intraperitoneale toediening, en vergeleken met die na toediening van ^{131}I/^{125}I-gelabeld MN-14. Opnieuw bleek dat intraperitoneale toediening leidde tot een hogere opname in tumorweefsel dan intraveneuze toediening. De opname van ^{88}Y-gelabeld MN-14 in tumor was hoger dan die van ^{186}Re- of ^{131}I/^{125}I-gelabeld MN-14, hetgeen waarschijnlijk wordt verklaard door verschillen in het lot van de radiolabels na afbraak van de gelabelde antilichamen in de tumoren. Op basis van dosimetrische analyse van de biodistributiegegevens werd berekend dat ^{131}I en ^{177}Lu de hoogste stralingsdosis aan tumoren konden afgeven. Radioimmunotherapie met ^{131}I- en ^{177}Lu-gelabeld MN-14 leidde inderdaad tot de hoogste mediane overlevingscijfers, vergeleken met ^{186}Re- en ^{90}Y-gelabeld MN-14. Op basis van de rsultaten van biodistributie- en therapiestudies werd geconcludeerd dat ^{131}I en ^{177}Lu de meeste geschikte radionucliden zijn voor radioimmunotherapie van kleine peritoneale metastasen.

Nadat het bovenbeschreven muizenmodel was gekarakteriseerd en geoptimaliseerd wat betreft antilichaamdosis, route van toediening, en radionuclide werd gezocht naar strategieën om de werking van radioimmunotherapie te versterken. Omdat er toenemend bewijs is dat remming van het cyclo-oxygenase (COX)-2 tumorcellen gevoeliger kan maken voor bestraling, werd verondersteld dat remming van het in de LS174T tumoren aanwezige COX-2 de werking van radioimmunotherapie zou kunnen versterken. In **hoofdstuk 5** worden de resultaten beschreven van studies waarin deze hypothese wordt getest. Hiertoe werd parecoxib, een pijnstiller speciaal ontwikkeld voor parenterale toediening. Parecoxib is een zogenaamde prodrug, welke na parenterale toediening wordt omgezet in het actieve metaboliet valdecoxib, de krachtigste COX-2 remmer die momenteel verkrijgbaar is. Parecoxib monotherapie bestaande uit dagelijkse intraperitoneale injecties van 0.2 – 25 mg/kg had geen meetbaar antitumor-effect. Parecoxib had ook geen effect op de biodistributie van ^{125}I-gelabeld MN-14. Toediening van parecoxib in combinatie met radioimmunotherapie, leidde tenslotte niet tot een verbetering van de overleving vergeleken met radioimmunotherapie monotherapie.

In de studies beschreven in **hoofdstuk 6** is een vergelijkbare poging gedaan om de werking van radioimmunotherapie te versterken, nu met behulp van gemcitabine, een frequent gebruikt cytostaticum voor chemotherapie van verschillende tumoren, en gekenmerkt door een sterk radiosensiterend vermogen. De keus van gemcitabine werd verder geinspireerd door een aantal publicaties waarin werd aangetoond dat gemcitabine de werking van radioimmunotherapie kon versterken in muizenmodellen van pancreascarcinoom (alvleesklierkanker) en coloncarcinoom. Na bepaling van de maximaal tolereerbare dosis van gemcitabine, bleek dat deze lager lag dan die geraporteerd in de literatuur, hetgeen mogelijk wordt verklaard door verschillen in muizenstammen. Uiteindelijk werden twee doseringsschema's getest. Het eerste schema bestond uit vier intraperitoneale injecties (0.11 mg of 0.33 mg/muis) op dag 0, 3, 6, en 9, waarbij de radioimmunotherapie (eenmalig) op dag 0 werd gegeven. In het tweede doseringsschema werd gemcitabine op vijf achtereenvolgende dagen toegedien (0.022 mg/muis), waarbij opnieuw de radioimmunotherapie eenmalig op dag 0 werd gegeven. In beide schema's, echter, leidde combinatietherapie niet tot een verbetering van de overleving, vergeleken met die na radioimmunotherapie alleen.

In **hoofdstuk 7** wordt de waarde van radioimmunotherapie als adjuvante behandeling onderzocht. Omdat cytoreductieve chirurgie niet haalbaar werd geacht in naakte muizen, werd hiervoor een reeds bestaand rattenmodel gebruikt. Peritoneale carcinomatose werd geinduceerd in Wag/Rij ratten door intraperitoneale injectie met tumorcellen van de ratten coloncarcinoom-cellijn CC-531. Gebruik werd gemaakt van het MG1 antilichaam, opgewekt in muizen geïmmuniseerd met CC-531 tumor cellen en gekenmerkt door weinig kruisreactiviteit met gezonde weefsels in Wag/Rij ratten. In een biodistributiestudie werd aangetoond dat de opname in tumor van MG1 gelabeld met ^{111}In (als surrogaat radionuclide voor ^{177}Lu) hoger was dan dat van MG1 gelabeld met ^{125}I

(als surrogaat radionuclide voor ^{131}I). Vervolgens werd het effect van radioimmunotherapie met ^{177}Lu-gelabeld MG1 onderzocht en vergeleken met dat van ongelabeld MG1 of een zoutoplossing. Radioimmunotherapie met ^{177}Lu-MG1 (74 MBq = 2 mCi per rat) had een uitgesproken antitumor effect, terwijl ongelabeld MG1 geen enkel antitumoreffect toonde. In de chirurgie-studie werden ratten met peritoneale carcinomatose behandeld met hetzij een exploratieve laparotomie (buikoperatie), een exploratieve laparotomie gevolgd door radioimmunotherapie met ^{177}Lu-MG1 (56 MBq = 1.5 mCi per rat), cytoreductieve chirurgie, of cytoreductieve chirurgie gevolgd door radioimmunotherapie. Cytoreductieve chirurgie resulteerde in een korte verlenging van de mediane overleving (10 dagen). Een exploratieve laparotomie gevolgd door radioimmunotherapie resulteerde in een verlenging van de mediane overleving van 21 dagen. Combinatie van beide behandelingen resulteerde uiteindelijk in een zeer significante verlenging van de mediane overleving van 47 dagen, hetgeen een additief effect van beide behandelingsmodaliteiten (cytoreductieve chirurgie en radioimmunotherapie) suggereert. Geconcludeerd werd dat radioimmunotherapie een effectieve adjuvante behandeling kan zijn na resectie van tumoren met een hoog risico op recidief, zoals na cytoreductieve chirurgie van peritoneale carcinomatose van colorectale origine.

In **hoofdstuk 8** wordt de hypothese getoetst dat het effect van adjuvante radioimmunotherapy na cytoreductieve chirurgie afhangt van het tijdstip van toediening van de gelabelde antilichamen. Wag/Rij ratten met kleine peritoneale metastasen werden hiertoe behandeld met hetzij een exploratieve laparotomie, cytoreductieve chirurgie, of cytoreductieve chirurgie gevolgd door radioimmunotherapie met ^{177}Lu-MG1 (56 MBq = 1.5 mCi per rat). De adjuvante radioimmuntherapie werd onmiddellijk, vier dagen, of veertien dagen na chirurgische cytoreductie gegeven. De chirurgische cytoreductie alleen resulteerde opnieuw in een korte verlenging van de overleving van tien dagen. Adjuvante radioimmunotherapie onmiddellijk na chirurgische cytoreductie, of vier dagen of veertien dagen later, resulteerde in een verlenging van de overleving van respectievelijk 48, 23 en 16 dagen. Het effect van adjuvante radioimmunotherapie na chirurgische cytoreductie was hiermee sterk afhankelijk van het tijdstip waarop de gelabelde antilichamen werden toegediend en nam snel af wanneer dit wordt uitgesteld. Geconcludeerd werd dat adjuvante radioimmunotherapie zo snel mogelijk na de cytoreductieve chirurgie moet worden gegeven.

In **hoofdstuk 9** worden de bovengenoemde resultaten bediscussieerd en wordt een perspectief geschetst voor toekomstig onderzoek naar de waarde van radioimmunotherapie voor de behandeling van peritoneale carcinomatose bij patiënten met een colon- of rectumcarcinoom.

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About the author

Manuel J. Koppe was born on December 24, 1973 in Kitwe, Zambia. In 1992 he graduated from Menso Alting College (Gymnasium), Hoogeveen. The same year he started his medical studies at the VU University, Amsterdam. During his studies he analyzed the results of surgical treatment for non-small cell lung cancer at the Netherlands Cancer Institute / Antoni van Leeuwenhoek hospital. After he graduated in 1999, he worked as a research physician at the Department of Surgery of the VU University Medical Center, Amsterdam, where he coordinated a clinical trial investigating the tumor targeting characteristics of ^{186}Re-labeled bivatuzumab in patients with breast cancer. Since 2000 he has worked as a surgical resident at the Department of Surgery of the Radboud University Nijmegen Medical Center. In close collaboration with the Department of Nuclear Medicine he designed the research program investigating the efficacy of radioimmunotherapy for the treatment of peritoneal carcinomatosis of colorectal origin, which resulted in the present thesis. In 2002 he received an AGIKO grant from the Netherlands Organization for Health Research and Development (ZonMw); in 2003 he was awarded the Prof. dr. P.J. Klopper Prize for the best surgical research proposal concerning adjuvant radioimmunotherapy after cytoreductive surgery in peritoneal carcinomatosis. In 2005 he started his surgical training at the Radboud University Nijmegen Medical Center (head: Prof. dr. R.P. Bleichrodt). Besides his surgical residency he will continue to be involved in surgical research. Manuel J. Koppe resides with his family in Nijmegen, The Netherlands.